

Idiopathic Menorrhagia: Clinical and Endometrial Effects of Local and Systemic  
Progestagens

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### Declaration

This thesis has been composed by myself, and I have been responsible for recruitment of patients, clinical management and laboratory studies unless otherwise acknowledged.

The contents of this thesis have not been submitted elsewhere for any other degree, diploma, or professional qualification.

Gillian A. Irvine,

March, 1997

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## Idiopathic Menorrhagia: Clinical and Endometrial Effects of Local and Systemic Progestagens

The complaint of excessively heavy periods, or menorrhagia, leads to a significantly reduced quality of life for many women. The definitive cure for menorrhagia is hysterectomy, however hysterectomy is unsuitable for many women and in these cases medical management is indicated.

The first part of this thesis reviews the possible aetiologies for idiopathic menorrhagia, discusses the investigation of patients complaining of abnormal menstrual bleeding, in particular the role of outpatient management, and summarises the medical therapies currently available with emphasis on the synthetic progestagens. Evidence for the involvement of the endothelins, a family of powerful vasoconstrictors, and nitric oxide, a vasodilator, in endometrial haemostasis is reviewed. The structural changes seen in the endometrium following exposure to synthetic progestagens are considered.

A review of 400 patients referred for outpatient hysteroscopy and endometrial biopsy to investigate abnormal vaginal bleeding is presented and its clinical implications discussed. Results from a randomised comparative parallel group study comparing the efficacy of systemic and local progestagens are presented. 44 patients with objectively proven idiopathic menorrhagia were randomised to receive oral norethisterone or local progestagens via the levonorgestrel intrauterine system. Outcome measures included the change in menstrual blood loss after three cycles of treatment, side effect profiles and patient satisfaction with treatment.

Endometrial biopsies were taken from patients participating in the above trial before and after treatment to examine the effects of exogenous progestagens on the endometrium. The following areas were investigated and are presented: changes in the distribution of endothelin A and B receptors; changes in the expression of endothelial and inducible nitric oxide synthase; the effects of exogenous progestagens on the luminal epithelium using scanning electron microscopy, and on morphological measurements of glandular and luminal epithelium under light microscopy; alterations to the secretory function of epithelial cells using *Dolichos biflorus* agglutinin histochemistry, and to the stromal cells using  $\alpha 2$  laminin, a marker of stromal cells which have undergone decidualisation.

## Chapter One

### A Review of the Literature

## Introduction

The human endometrium is a remarkable organ. In the absence of pregnancy, it undergoes a cycle of shedding (menstruation), remodelling, cellular proliferation and secretory change, all within an average of 28 days. In the event of pregnancy, the endometrium is able to postpone menstruation, establish physical and nutritional contact with the conceptus and facilitate implantation. Ovarian steroids exert overall control over the changing functions of the endometrium and the endometrium also functions as a paracrine organ, with the different compartments of the endometrium interacting with each other via a number of mediators. Abnormalities of endometrial function can result in menstrual disturbances such as excessively heavy menstrual blood loss (menorrhagia), the subject of this thesis, and abnormal endometrial development in the luteal phase is associated with unexplained infertility (Li et al, 1991) and carries a poor prognosis for subsequent pregnancy (Klentzeris et al, 1992).

The first part of this review will examine the structural changes occurring in the endometrium during the normal menstrual cycle, in early pregnancy and following exposure to exogenous progestagens. Alterations in endometrial function will also be examined, with particular emphasis on endometrial haemostasis and the changes associated with stromal decidualisation. The potential roles of endothelin and nitric oxide in these processes will be highlighted.

The second part of this review will consider clinical aspects of menorrhagia. The clinical definition is reviewed and the aetiology discussed. The medical treatment of menorrhagia is assessed, with emphasis on the role of synthetic progestagens in the reduction of menstrual blood loss.

## Structural and Functional Changes in the Endometrium

Within the endometrium, four cellular compartments may be recognised, the lining surface epithelium, the endometrial glands, stroma and blood vessels. All respond to a changing pattern of ovarian steroids, with cyclical alterations in the non-pregnant state and a pattern of progesterone dominance in the pregnant state. The response to the ovarian steroids varies between compartments and is mediated via intracellular oestradiol (ER) and progesterone receptors (PR). The concentrations of both receptors increase during the proliferative phase, the rise in PR lagging behind that of ER (Lessey et al, 1988), and return to low levels during the secretory phase. Immunohistochemical studies have identified maximal ER expression in endometrial glands and stroma in the late proliferative phase (Snijders et al, 1992). Progesterone receptors show maximal expression in glands and stroma in late proliferative phase endometrium, with stromal staining persisting into the secretory phase of the cycle. Three distinct layers can be identified in the endometrium, a superficial zona compacta, an intermediate zona spongiosa and the zona basalis next to the myometrium. Together the zona compacta and spongiosa make up the functional layer of the endometrium (stratum functionalis), which undergoes more dramatic changes than the deeper layer (stratum basalis) during the menstrual cycle. This section reviews the response of the four endometrial compartments to endogenous and exogenous steroids.

### Endometrial glands and stroma: an overview

The histological changes occurring within the endometrium during the menstrual cycle are well-established. Work published in 1950 by Noyes, Hertig and Rock reviewed

the criteria used to date an endometrial biopsy and thus provided a detailed description of the morphological changes occurring within endometrial glands and stroma in approximately 8 000 endometrial biopsies (Noyes et al, 1950).

In the early proliferative phase, Noyes et al described the endometrial glands as short, straight and narrow with mitoses indicating proliferation; the stroma was compact. In the mid proliferative phase the glands had become longer and were lined with columnar epithelial cells. Transient stromal oedema was noted and stromal mitoses were common. In the late proliferative phase the glands were described as tortuous with active growth and pseudostratification of the epithelium. The stroma was moderately dense and actively growing. During the secretory phase, changes in endometrial glands and stroma were more marked than in the proliferative phase, such that the authors felt able to categorise changes on a daily basis. In the first part of the secretory phase, these changes were mainly confined to the glandular compartment. By the 16th day of the cycle the glands showed subnucleolar vacuolation, and by day 17 the gland nuclei were lined in a more or less orderly row with homogeneous cytoplasm above the nuclei and large vacuoles below. By day 18 the vacuoles had decreased in size as their contents entered the gland lumen, and the nuclei had approached the base of the cell. By day 19 there were few vacuoles remaining and by day 20 the gland lumina were seen to contain a central collection of secretion. Changes in the rest of the secretory phase were mainly categorised by changes occurring in the stromal compartment. Tissue oedema was evident by day 21, reaching its peak on day 22. By day 23 the spiral arterioles had become more prominent and periarteriole stromal cells underwent early decidual change with larger nuclei and an increase in cytoplasm. By day 24 decidual cells were evident around the arterioles, and mitoses were visible within the stroma. By day 25 decidual differentiation had occurred below the surface epithelium, and by day 27 appeared as solid sheets of well developed decidua-like cells. Infiltration by polymorphonuclear

leukocytic cells began on day 27 and areas of focal necrosis and haemorrhage were seen to occur a few hours before the onset of overt menstruation.

#### Endometrial glands and secretory markers

This work, while including very large numbers of biopsies, has been criticised because it used subjective methods to assess the tissue and the date of the last menstrual period to date the biopsy (Li et al, 1988). Other studies have undertaken objective morphometric analyses of endometrial glands and stroma, and have used the day of the luteinising hormone (LH) surge as a reference point (Johannisson et al, 1987; Li et al, 1988). Measurements of the functional layer of endometrial biopsies in 90 women with regular menstrual cycles showed that the number of glands per mm<sup>2</sup> was 20 and that this did not vary over the course of the cycle (Johannisson et al, 1987). Glandular mitoses, counted per 1000 glandular cells and expressed per 48 hours, remained stable from days LH -11 /-10 to days LH -5 /-4, then decreased significantly from days LH -3 /-2 to days LH +5 /+6 suggesting a reduction in proliferative activity as ovulation approached. Glandular lumen diameter remained constant throughout the proliferative phase, showed a highly significant linear increase by day LH +11 /+12 while gland cell height decreased significantly from days LH +3 /+4 to the end of the cycle. Li and co-workers described in detail the events occurring in the days following the LH surge (Li et al, 1988), and showed a highly significant correlation between five morphometric measurements (number of mitoses per 1000 gland cells, amount of secretion in the gland lumen, volume fraction of gland occupied by gland cell, amount of pseudostratification of gland cell and amount of decidual reaction) and chronological dating based on the LH surge. The importance of progesterone in effecting secretory change within the endometrium was emphasised in a study in which an anti-progestin was administered to women two days following the



LH surge (Cameron et al, 1996). This resulted in an endometrium with retarded secretory changes compared to a control cycle, and immunostaining demonstrated persistent ER and PR staining in glands and stroma following treatment.

In addition to light microscopy studies of glandular epithelium, molecular changes occurring in the glycoproteins associated with glandular epithelial cells are of interest since such changes respond to changes in the function of such cells. Luminal and glandular epithelium is associated with a thick glycocalyx at the apical surface of the cells which may play a role in implantation. Lectins are haemagglutinins isolated from a variety of plant and animal sources with combining sites specific for carbohydrates, and several lectins have been demonstrated to bind selectively to endometrial epithelial cells and their secretions (Aplin, 1991). Using a panel of lectins, Aoki and colleagues demonstrated that the incidence and intensity of staining for *Dolichos biflorus* agglutinin (DBA) and soybean agglutinin (SBA) increased in glands in secretory phase endometrium (Aoki et al, 1989) and that the reaction product of DBA localised to the Golgi apparatus in the supranuclear region of the glandular epithelial cell. DBA staining can be detected at the apical surface of glandular epithelial cells and in intraluminal secretions in proliferative phase endometrium, but expression is increased following ovulation, suggesting up-regulation from a basal level of secretory activity (Aplin, 1991).

#### Endometrial stroma and decidualisation

Decidualisation of the stroma is central to both key functions of the endometrium, implantation of the developing blastocyst and menstruation. Decidualisation is found in species where the trophoblast penetrates the stroma to gain access to the maternal vasculature. This may occur in response to the invading blastocyst, as in rodents, or



independent of a stimulus from the blastocyst, as in primates. Menstruation is restricted to fewer species than decidualisation, and appears to occur only in species in which decidualisation is part of the normal menstrual cycle (Finn, 1987). Three components of endometrial stroma are considered further here, stromal cells, extracellular matrix and cellular basement membrane, all of which undergo changes in response to the changing pattern of ovarian steroids during the normal menstrual cycle and in pregnancy.

*Stromal cells: fibroblasts / decidual cells*

The endometrial stroma contains mesenchymal cells. During the proliferative phase of the cycle the major cellular component is the fibroblast. These show a slow steady growth, the number of stromal mitoses per 1000 stromal cells expressed per 48 hours showing a significant increase between days LH -11 /-10 to LH +1 /+2 (Johannisson et al, 1987). Following ovulation and exposure of the endometrium to progesterone, the volume density of stroma decreases as the volume density of the glandular compartment increases. The main changes in the stroma occur in the mid- to late secretory phase, when the stroma becomes oedematous and decidual cells are apparent, first appearing in peri-arterial and subluminal areas (Noyes et al, 1950). Under light microscopy, decidual cells exhibit a vesicular nucleus and abundant clear cytoplasm. Ultrastructural studies of the decidual cells have demonstrated that the cells are larger than the stromal cells of the early secretory phase with rounded nuclei, dilated rough endoplasmic reticulum and more prominent Golgi bodies and lysosomes (Verma, 1983; Cornille et al, 1985). Intermediate filaments accumulate as decidual cells differentiate and provide information about their cellular origin with both rat and human studies demonstrating expression of vimentin and desmin by decidual cells, suggesting that they are derived from stromal fibroblasts (Glasser and Julian, 1986; Khong et al, 1986).

### *Stromal cells: large granular lymphocytes*

The endometrial stroma contains a unique population of granulated cells. Similar to the natural killer group of T lymphocytes, surface markers show them to be a distinct population of CD3<sup>-</sup>, CD56<sup>+</sup> cells (Starkey et al, 1988; King et al, 1989) which increase significantly due to cell proliferation in the late secretory phase of the cycle (King et al, 1989) and in early pregnancy (King and Loke, 1991). Their precise function is unknown, but they may play a role in controlling the extent of trophoblast invasion (King and Loke, 1991).

### *Extracellular matrix*

The stromal extracellular matrix consists of collagens I, III, V, VI and fibronectin (Aplin et al, 1988). During the menstrual cycle, changes in the organisation of the extracellular matrix occur in response to changes in plasma steroid levels. From day 21 of the cycle the matrix becomes more oedematous, with the appearance of collagen V epitopes suggesting collagen breakdown and also a reduction in the content of collagen VI which bridges interstitial structures of connective tissues such as collagen fibrils, nerves and blood vessels (Aplin et al, 1988; Keene et al, 1988; Bonaldo et al, 1990). Expression of type VI collagen mRNA is constant in whole endometrial tissue throughout the menstrual cycle, suggesting that the reduction in collagen VI is due to breakdown or reorganisation of fibrils rather than a reduced production (Mylona et al, 1995). This matrix reorganisation is thought to facilitate implantation.

### *Cellular basement membrane*

Early ultrastructural studies demonstrated that decidual cells were associated with a pericellular matrix which resembled a pericellular basement membrane (Lawn et al, 1971). Decidual cells have been demonstrated to produce a capsular basal lamina

containing laminin, collagen type IV, heparan sulphate proteoglycan and the glycoprotein BM-40 (Wewer et al, 1985; Faber et al, 1986; Aplin et al, 1988). During the proliferative phase of the cycle, no production of basal lamina components was observed (Wewer et al, 1985), but in early secretory endometrium immunoreactive laminin was detectable before histological evidence of decidualisation (Faber et al, 1986). Using a panel of monoclonal antibodies specific for various laminin subunits, pregnant stromal basement membrane was demonstrated by immunofluorescence and Western blotting to contain laminin subunits  $\alpha 2$ ,  $\beta 1$ ,  $\beta 2$  and  $\gamma 1$  (Church et al, 1996). During the nonpregnant cycle  $\alpha 2$  laminin was absent from proliferative tissue, but was present in the late secretory phase in areas of decidualisation. RT-PCR demonstrated that  $\alpha 2$  laminin chain mRNA was present in decidua, with small amounts of this transcript detectable in endometrial tissue throughout the cycle, suggesting that production of  $\alpha 2$  laminin is under hormonal control. The function of the decidual pericellular basement membrane is unknown, but it may play a role in controlling the adhesion, migration, and differentiation of invading trophoblast cells (Church et al, 1996).

### Surface epithelium

The surface epithelium is the first interface between maternal and foetal tissues and lends itself to scanning electron microscopy studies of its structure, as well as conventional histological studies by light microscopy.

The changes occurring in the surface epithelium during the menstrual cycle have been well documented (Johannisson and Nilsson, 1972; Ferenczy, 1976; Ludwig et al,

1990). Twenty four hours after the onset of menses the uterine cavity shows areas of desquamation over most of its surface, but the basal stumps of the glands remain. On the second and third day after the onset of menses, epithelium grows out from the basal glands over the epithelial stroma which is partly covered by fibrin mesh. Macrophages can be identified clearing the uterine slough, with a peak of activity on the third and fourth days. By the fifth and sixth day of the cycle the endometrium has completely re-epithelialised, and further differentiation of the surface epithelium begins with ciliogenesis and the formation of microvilli. Ciliated cells are predominantly found around the gland openings, with increasing numbers of ciliated cells towards the endocervix. Surface microvilli are also identified. By the mid-secretory phase the cilia are showing signs of degeneration and the microvilli become adherent to each other. In early pregnancy the surface of the uterus shows an absence of cilia and small, irregular microvilli (Johannisson and Nilsson, 1972). In a study of 68 women undergoing curettage, the percentage of ciliated cells in glandular and luminal epithelium was demonstrated to rise during the proliferative phase to a maximum of 20% at the time of ovulation (Masterton et al, 1975). During the secretory phase of the cycle, this percentage declined. It has been suggested that cilia are important for the transport of secretory products, and their position around gland openings (Johannisson and Nilsson, 1972) gives support to that hypothesis. The reduction in the percentage of ciliated cells during the secretory phase of the cycle (Masterton et al, 1975), along with their degeneration (Johannisson and Nilsson, 1972) at a time when secretions are only starting to appear in the gland lumina (Noyes et al, 1950) suggests that they may have another purpose such as ovum or sperm transport.

#### Endometrial vasculature and menstruation

The endometrium is supplied by spiral arterioles arising from basal arteries in the myometrium. These vessels regrow from the vascular stumps remaining in the

stratum basalis following menstruation into markedly coiled end arterioles, each supplying 4-7mm<sup>2</sup> of the endometrial surface (Markee, 1940). Vessels of the myometrium and basal layer of the endometrium are little influenced by cyclical hormone changes, but vessels of the functional layer do take part in the cyclical endometrial changes.

The physiological changes that occur in the endometrium during menstruation were first documented over 50 years ago, with work by Markee looking at endometrial ocular explants (Markee, 1940) and histological studies by Bartelmez (Bartelmez, 1933). Other work has included histological studies by Christiaens of hysterectomy specimens taken within 72 hours of the onset of menses (Christiaens et al, 1980), scanning electron microscopy studies by Ludwig (Ludwig et al, 1990) and in vitro models for the regulation of peri-implantational haemostasis and menstruation described by Lockwood and Schatz (Lockwood and Schatz, 1996).

Decidual transformation of stromal cells begins in peri-arterial areas, before spreading throughout the endometrium (Noyes et al, 1950). Plasminogen activators (PAs) initiate conversion of plasminogen to plasmin, which degrades extracellular matrix proteins as well as fibrinogen. In a decidualisation model based on stromal cell monolayers derived from decidualised endometrium, the decidualised stromal cells are associated with reduced expression of urokinase PA, tissue type PA (Schatz et al, 1995) and the extracellular matrix degrading matrix metalloproteinase (MMP-3) (Schatz et al, 1994), and increased expression of plasminogen activator inhibitor (PAI-1) (Schatz et al, 1995) and tissue factor (TF), an initiator of coagulation (Lockwood et al, 1993). Thus decidualised stromal cells are well placed to control bleeding from blastocyst invasion by promoting a state of increased haemostasis and decreased fibrinolysis.

Menstruation is preceded by regression of the endometrium. Withdrawal of steroids from the decidualisation model resulted in reduced levels of TF and PAI-1, thus promoting fibrinolysis and the degradation of extracellular matrix proteins by plasmin and MMP-3, which is activated by plasmin (Lockwood et al, 1994; Lockwood et al, 1995). Degradation of the extracellular matrix leads to an increased coiling of the uterine spiral arterioles. Immediately prior to the onset of menses, intense spiral arteriole vasoconstriction occurs (Markee, 1940). When these vessels dilate, menstrual bleeding occurs, with blood escaping from the ends of damaged arterioles directly into the uterine cavity, or through the vessel walls to form endometrial haematomata (Christiaens et al, 1980). Some bleeding also occurs from damaged veins, and red cells may pass through the walls of vessels by diapedesis. Patchy loss of endometrial tissue follows, with successive layers being lifted off as bleeding continues. Haemostatic mechanisms in the endometrium are strikingly different to vessels elsewhere in the body. Platelet activation and adhesion in endometrial vessels is initially suppressed, but with increased blood extravasation, damaged vessel ends are sealed by intravascular plugs of platelets and fibrin. A morphometric study of hysterectomy specimens in the early menstrual phase of patients with proven menorrhagia showed a positive correlation between menstrual blood loss and the number of haemostatic plugs, the authors suggesting that these plugs are more fragile and easily displaced in menorrhagic women than in controls (van Eijkeren et al, 1991). By 20 hours after the onset of menses, when most of the endometrial shedding has occurred, haemostasis is achieved by a further intense spiral arteriole vasoconstriction. Tissue regeneration begins within 36 hours of the onset of menses, while some endometrial shedding is still occurring, and arises from glands in the basal layer of the endometrium (Ludwig et al, 1990). As well as changes in the endometrium, events also occur in the uterine cavity, with coagulation and rapid anticoagulation of menstrual blood as it passes through the uterine cavity (Sheppard et al, 1983; Rees et al, 1985).

### The onset of menstruation

Stromal regression prior to menstruation is a consistent finding in many histological studies of the normal menstrual cycle (Noyes et al, 1950; Johannisson et al, 1987; Li et al, 1988) and has also been noted in an experimental mouse model (Finn and Pope, 1984). There is some debate about whether the trigger for menstruation is regression of the decidualised stroma, with the resulting shear stresses leading to the release of vasoactive substances, or whether spiral arteriole vasoconstriction is the primary event, with stromal changes occurring secondary to altered blood flow. A variety of vasoactive agents have been implicated in pre-menstrual vasoconstriction including prostaglandin F<sub>2</sub> $\alpha$  (Abel and Baird, 1980) and the endothelins, which will be discussed in more detail later in this chapter.



## The Effects of Synthetic Progestagens on the Endometrium

The effects of synthetic progestagens on the endometrium have been examined in a number of studies, using a variety of different progestagens, administered by a variety of methods, and with a variety of exposure times. These studies have been mainly concerned with contraceptive regimens of progestagens and many focused on the breakthrough bleeding associated with synthetic progestagen administration, since this represents a common clinical problem which can limit the acceptability of the method. The effects of systemic and local administration of synthetic progestagens on the compartments of the endometrium are summarised below.

### Systemic progestagen administration

Progestagens may be administered orally, via long-acting intramuscular injection or more recently via delivery systems such as vaginal rings or subcutaneous implants.

#### *Endometrial glands*

Following administration of different types of oral progestagens for a minimum of two months, endometrial glands showed a reduction in the number of mitoses and reduced tortuosity (Ludwig, 1982). Pseudostratification was a common finding, but basal vacuolation was reduced. Longer term use resulted in a scant endometrium with large glands. Injection of depot medroxyprogesterone acetate resulted in a reduction in gland cell height from the 20th day after injection (Roberts et al, 1975). Vaginal rings releasing levonorgestrel produced a significant reduction in gland diameter after six weeks of use and this effect was dose dependent (Johannisson et al, 1982). In a study



using different doses of intramuscular progesterone as part of hormone replacement therapy (HRT) in women with premature ovarian failure, low dose progesterone was associated with an increase in glandular mitoses and in the volume fraction of gland occupied by gland cells compared with a standard regimen, and increasing the dose of progesterone did not change these parameters (Li et al, 1992).

### *Stroma*

Synthetic gestagens had a marked effect on the stroma in a number of studies (Landgren et al, 1979; Ludwig, 1982; Li et al, 1992). Stromal oedema increased in a focal pattern and decidualisation was a common finding following oral administration of a number of different progestagens (Ludwig, 1982). This was proportional to the duration of progestagen use and leukocyte infiltration of the stroma was apparent. The decidual reaction was shown to be proportional to the dose of progestagen in a study using vaginal rings as the vehicle for norethisterone release (Landgren et al, 1979). During a dose-finding HRT study, high dose intramuscular progesterone resulted in a significant increase in stromal cell diameter on day 19 of the cycle, while producing no changes in endometrial glandular morphology (Li et al, 1992).

### *Surface epithelium*

Defective ciliogenesis was a common response to oral treatment and was observed in all samples of endometrium following at least two months exposure to oral treatment (Ludwig, 1982). A reduction in size and numbers of microvilli was apparent as soon as 5 days after injection with depot medroxyprogesterone acetate (Roberts et al, 1975).

Long term use of oral progestagens resulted in large venous vessels, often including vessels in the superficial layer of the endometrium (Ludwig, 1982). This was confirmed in the endometrium of women exposed to oral norethisterone or medroxyprogesterone acetate for between eight days and six months when dilated venules were observed in progestagen-exposed but not in control samples, and were more often seen in specimens strongly suppressed by progestagen or those with atrophic changes (Song et al, 1995). A decrease in microvascular density was demonstrated using immunohistochemical staining for von Willebrand factor and CD34 in progestagen-exposed biopsies. In contrast a detailed study of the endometrial microvascular density following exposure to subcutaneous levonorgestrel for between three and 12 months showed an increase in the endometrial microvascular density compared with controls (Rogers et al, 1993). Subsequent work demonstrated both a reduction in endometrial endothelial cell proliferation in endometrium exposed to subcutaneous levonorgestrel (Goodger (MacPherson) et al, 1994) and a reduced endothelial cell migratory response to endometrial explants from subcutaneous levonorgestrel users compared with normal controls (Subakir, 1995). These results suggest that the observed increase in microvascular density in endometrium exposed to subcutaneous levonorgestrel may be due to a lower rate of regression in the vascular compartment compared to the stromal and glandular compartments.

#### Intrauterine progestagen administration

Synthetic progestagens can be administered directly to the endometrium via a hormone-impregnated intrauterine device. First developed for contraception (Pharriss et al, 1974), such devices are now being used for therapeutic indications such as the treatment of menorrhagia (Milsom et al, 1991), and have a potential role in the

hormone replacement therapy and the treatment of endometrial hyperplasia. The first report of the intrauterine administration of a progestagen in humans was by Scommegna in 1970, who introduced a device releasing 30-40 $\mu$ g progesterone into the uterine cavity of 13 women between 18 hours and seven days prior to hysterectomy (Scommegna et al, 1970). The endometrium was found to have been converted from a proliferative to a secretory pattern after as little as 18 hours exposure. Over the next few days of exposure, Scommegna reported a discordance in maturation of the endometrial glands and stroma, with the stroma being more advanced than the glands. After 24 hours exposure decidualisation was noted, and by the fourth day of exposure the glands began to undergo involutional change. It was noted that, with this device, the functional layer was more affected than the basal layer.

In a dose-finding study using 4 different release rates of progesterone from 20-110 $\mu$ g / 24 hours, biopsies were taken following two to ten months of exposure (Martinez-Martinou et al, 1975). All biopsies showed evidence of variation in the normal endometrial pattern, varying from a secretory appearance to a suppressed endometrium, with the higher release rates tending to endometrial suppression. Decidual reaction was common, particularly in the suppressed endometria. An inflammatory infiltrate was noted in half of the biopsies. When a device releasing 65 $\mu$ g progesterone in 24 hours (Progestasert<sup>®</sup>, Alzo Corp, Palo Alto, Ca., USA) was studied, the response to the device was said to be decidual in nature, initially at its most intense immediately adjacent to the delivery system (Pharris, 1977). Later it was noted to spread out to the distal endometrium. Glands were described as small and few, lined with cuboidal rather than columnar cells.

Scanning electron microscopy studies of the endometrium exposed to the Progestasert<sup>®</sup> showed fewer shorter ciliated cells (Bonnar and Sheppard, 1979) and a

reduction in the surface micro-villi (Gonzalez-Augulo et al, 1979). Erosion of the surface epithelium, was noted below the inert arms of the device but to a lesser degree than that found with inert or copper containing devices (Bonnar and Sheppard, 1979).

Studies of the vasculature following exposure to Progestasert® showed capillary microthrombosis within the functional layer of endometrium adjacent to the inert arm of the device (Sheppard and Bonnar, 1980). This is similar to that found with inert or copper-containing intrauterine contraceptive devices, but was noted to be less with progesterone releasing devices. Dilated venules were a common finding.

Studies using a more potent progestagen, D-norgestrel, showed a more profound and uniform effect on the endometrium (Nilsson et al, 1978). Two groups of patients were studied, those having an endometrial biopsy taken after a median of 96 days exposure (range 74-118 days) and those undergoing hysterectomy after 29 to 36 days exposure. The endometrial biopsies showed a pattern of scarce glands with a low epithelium and no signs of mitotic activity, set within an oedematous decidualised stroma. An inflammatory infiltration was noted. Hysterectomy specimens confirmed that the changes affected all layers within the endometrium and that the whole of the uterine cavity was affected, not just that endometrium in contact with the device, suggesting that intrauterine D-norgestrel had a more uniform and profound effect on the endometrium than intrauterine progesterone. Non-invasive studies of the effects of intrauterine levonorgestrel on endometrial thickness and uterine blood flow using transvaginal ultrasound and Doppler blood flow demonstrated a reduction in endometrial thickness by ten weeks after insertion, but no alteration in uterine blood flow (Pakarinen et al, 1995).

This section has reviewed the structural and functional changes occurring within the endometrium during the normal menstrual cycle and in response to exogenous

progestagen administration. The next section reviews the evidence supporting a role for nitric oxide and the endothelins as mediators involved in paracrine and autocrine interactions in the endometrium.

## Nitric Oxide and Nitric Oxide Synthase

Nitric oxide (NO), a noxious, free radical species, has long been recognised as an atmospheric pollutant. Within the past decade however, understanding of the biological roles of this simple molecule has mushroomed. Work investigating its actions has been carried out in several different fields of biological research simultaneously, such that NO was awarded the title "Molecule of the Year" by the journal Science in 1992. In 1987 it was shown that mammalian vascular endothelial cells could produce the molecule (Palmer et al, 1987). Since then, NO has been shown to be an important messenger in a number of diverse mammalian systems.

### Synthesis and Cellular Actions of Nitric Oxide

NO is produced from the precursor L-arginine, catalysed by the enzyme nitric oxide synthase (NOS). This was demonstrated in studies showing that NO was released from porcine endothelial cells in the presence of L-arginine and that the addition of  $^{15}\text{N}$ -labelled L-arginine produced  $^{15}\text{NO}$  (Palmer et al, 1988). Simultaneous work in the field of immunology demonstrated that activated murine macrophages could metabolise L-[guanido- $^{15}\text{N}_2$ ] labelled arginine to  $^{15}\text{NO}_2^-$  and  $^{15}\text{NO}_3^-$  (Stuehr and Marletta, 1985) and that activated macrophages were able to inhibit tumour cells in the presence of L-arginine but not D-arginine, with L-citrulline and nitrite ( $\text{NO}_2^-$ ) being produced as by-products (Hibbs et al, 1987).

Nitric oxide exerts its effects by the activation of soluble guanylate cyclase, leading to an increase in the concentration of cyclic guanosine monophosphate (cGMP) in target cells (Lowenstein and Snyder, 1992).

Three classical areas of NO function have been described. These are concerned with vascular effects, cell mediated immunity and neurotransmission in the brain and peripheral nervous system. It is interesting to bear in mind that research in these three fields was taking place simultaneously, with developments in one field opening up lines of research in another.

### Vascular functions

One of the first description of the molecule's effects was in the area of vascular physiology. Earlier work had demonstrated that acetyl choline could produce relaxation of blood vessels only in the presence of an intact endothelium, which produced a diffusible substance known as endothelium derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980). Further studies suggested that NO and EDRF were one and the same (Furchgott, 1988). This was confirmed with the observations that both substances had the same physiological activity, the same short biological half life (less than five seconds), and that both were inhibited by haemoglobin and enhanced by superoxide dismutase to a similar degree (Palmer et al, 1987). In addition, both EDRF and NO were shown to have similar effects on platelet function, inhibiting platelet aggregation (Radomski et al, 1987a) and adhesion to the endothelium (Radomski et al, 1987b).

### Cell mediated immunity

It has long been recognised that macrophages, activated by cytokines released from sensitised lymphocytes, form the basis of cell mediated immunity and mount a non-specific response to bacteria, protozoa or tumour cells. The role of NO in cell



mediated immunity was first proposed in 1987 (Hibbs et al, 1987). Since then, NO mechanisms have been demonstrated in the cell mediated response to a number of micro-organisms (Granger et al, 1986; Adams et al, 1990; Liew et al, 1990), with the suggestion that the L-arginine / NO pathway may have originally evolved as a first line defence against intracellular parasites or neoplastic cells.

### Neurotransmission

A number of agents have been shown to play a part in neurotransmission in the central nervous system, a common feature of many being an associated increase in cyclic GMP levels. In 1977, it was reported that NO could stimulate guanylate cyclase in homogenates of mouse cerebral cortex (Miki et al, 1977). Following the identification of the L-arginine / NO pathway in vascular physiology, it was demonstrated that the addition of L-arginine to rat synaptosomal cytoplasm in the presence of NADPH resulted in the formation of NO and citrulline and was accompanied by the stimulation of soluble guanylate cyclase (Knowles et al, 1989). This reaction was inhibited by L-NMMA, suggesting that rat neural tissue contained NOS. Rat cerebellar cells were also shown to produce an EDRF-like material which was associated with a rise in cyclic GMP levels when N-methyl D aspartate (NMDA) receptors were stimulated (Garthwaite et al, 1988). NO has also been shown to play a role in peripheral neurotransmission via the non-adrenergic non-cholinergic (NANC) nerves, an important component of the autonomic nervous system (Rand, 1992).



Nitric Oxide Synthase

NO is not stored in cells, but is synthesised on demand. Inactivation or reuptake mechanisms have not yet been discovered and its biological activity is therefore regulated by its production and by its rate of clearance. The production of NO is catalysed by the enzyme nitric oxide synthase (NOS), which oxidises the guanidine group of L-arginine in the presence of NADPH. A number of cofactors are involved in the reaction, which also requires the presence of calmodulin. The clearance of NO is catalysed by the enzyme superoxide dismutase. The production of NO is summarised in figure 1.1.

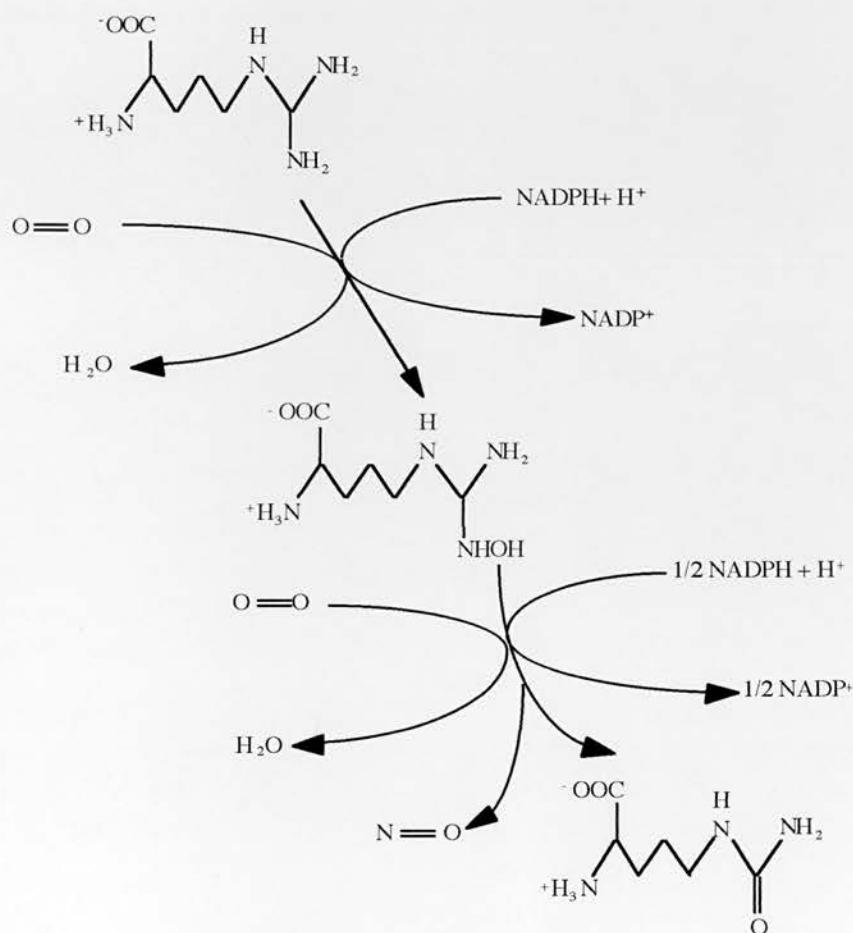


Figure 1.1. The production of NO.

NO is a labile molecule and its direct measurement is difficult. Measuring the conversion of L-arginine to L-citrulline is one method of inferring the production of NO in tissues, while the oxygenation products of NO (nitrites and nitrates) can be measured following reduction of nitric oxide with nitrate reductase using the Greiss reaction. With the sequencing and purification of NOS enzymes, detection of NOS mRNA and protein in tissues is now possible using a variety of techniques including immunohistochemistry, Western blotting, Northern analysis, in situ hybridisation and the reverse transcription-polymerase chain reaction (RT-PCR). Three different isoforms of NOS have been characterised, first described in brain tissue, endothelial cells and macrophages. They are characterised in part by their sensitivity to calcium.

#### *Type I or neural NOS*

Rat neural NOS (nNOS) was the first form to be described. The enzyme was found to be expressed at high levels in the brain, in particular the cerebellum, and was dependent on calcium and calmodulin for its activity (Bredt and Snyder, 1990).

#### *Type II or endothelial NOS*

NOS from vascular endothelial cells (eNOS) was also constitutively expressed and calcium/ calmodulin - dependent (Moncada et al, 1991). Initially it was assumed that the nNOs and eNOS were the same, but cloning of both bovine and human constitutive NOS's has shown them to be two distinct gene products, sharing 57% amino acid homology.

#### *Type III or iNOS*

NOS activity in rat macrophages was found to be different from the other two isoforms. Under basal conditions enzyme activity was negligible, but with stimulation by lipopolysaccharide and certain cytokines, production was massively enhanced

within a few hours (Stuer and Marletta, 1987). This enhancement was independent of the presence of calcium (Nathan and Hibbs, 1991).

The distinction between the three different isoforms is recognised now to be more complex than was originally thought, with evidence of calcium-dependent inducible enzymes (Palmer et al, 1992) and inducible constitutive forms (Salter et al, 1991).

The biological effects of the various isoforms of NOS were demonstrated with the successful breeding of mice with the different isoforms of NOS inactivated through gene knockout techniques. Null mutant nNOS (nNOS<sup>-</sup>) mice were noted to have dilated stomachs with constricted pyloric sphincters (Huang et al, 1993), and appeared to be resistant to brain damage caused by vascular strokes (Huang et al, 1994). iNOS<sup>-</sup> mice were noted to have reduced defences against intracellular pathogens such as *Listeria* and *Leishmania* and were more susceptible to lymphoma; these mice were more successful at resisting the hypotension associated with endotoxic shock and other inflammatory stimuli (MacMicking et al, 1995). eNOS<sup>-</sup> mice exhibited systemic hypertension confirming the importance of NO as a physiological vasodilator (Huang et al, 1995).

*yes, need ref*

## Nitric Oxide and Nitric Oxide Synthase in the Uterus

A number of studies in both animal and human tissue have identified the involvement of a nitric oxide-cGMP system in several areas of uterine physiology and are summarised here. While animal studies offer a convenient method of studying NO and NOS in the uterus, care should be taken when drawing comparisons between species. Investigations into the role of NO in the uterus have concentrated on three main areas concerned with myometrial contractility, vasodilatation and neurotransmission.

### *Myometrial contractility*

The effects of the molecule on smooth muscle led many workers to investigate the possibility that NO is responsible for maintaining a quiescent uterus during pregnancy. This theory was supported by anecdotal reports of the use of NO donors to induce uterine relaxation for procedures such as manual removal of placenta (Peng et al, 1989) or to correct uterine inversion (Bayhi et al, 1992) and by an observational study which suggested that glyceryl trinitrate arrested preterm labour (Lees et al, 1994).

L-arginine, NO and sodium nitroprusside (a NO donor) were shown to inhibit the inherent contractility of rat uterine muscle strips taken during pregnancy, delivery and post partum (Yallampalli et al, 1993). L-NAME, an inhibitor of NOS, increased contractility while methylene blue, an inhibitor of soluble guanylate cyclase, and L-N<sup>G</sup>-nitro-arginine, a specific blocker of NOS, prevented the relaxation effects of L-arginine. Measurement of uterine nitrates and nitrites showed that levels were significantly higher preterm than at term and the inhibitory effects of L-arginine were reduced in tissues of animals at term compared with earlier in pregnancy, suggesting that NO-induced relaxation declines towards delivery and that a NO system maintains

uterine quiescence during pregnancy but not during delivery (Yallampalli et al, 1993). This theory was supported by rabbit studies (Sladek et al, 1993), when it was demonstrated that NOS activity, as measured by an L-arginine conversion assay and by the Greiss reaction, in uterine homogenates was higher in the decidual fraction than the myometrial fraction, and decidual NOS activity was highest on day 27, declining to one fifth of this value by term. NOS activity was not calcium-calmodulin dependent, the authors suggesting a role for iNOS in producing an endogenous uterine relaxant (NO) which decreases towards term. Guinea pig studies showed that myometrial cGMP increased abruptly half way through pregnancy to 200 times the non-pregnant value, declining sharply again towards the end of pregnancy (Weiner et al, 1994). However when measurements were made of both calcium-dependent and calcium-independent NOS activity during pregnancy (using an L-arginine conversion assay), although they found a decline in myometrial NOS activity with advancing gestation, there was no difference in NOS activity when compared to non-pregnant animals. The authors proposed that, in the guinea pig, the mechanism for increasing myometrial cGMP was independent of NOS.

The effects of exogenous steroids on uterine NO production were examined when full thickness uterine tissue from prepubertal rats treated with exogenous oestradiol or oestradiol and progesterone showed a significant reduction in nitrite and nitrate production, as measured by the Greiss reaction, when compared with control and progesterone-treated rats (Yallampalli et al, 1994). The authors suggested a steroid hormone influence on the production of NO in the rat uterus. By contrast, a study in the sheep found that administration of oestradiol increased NOS activity in the myometrium, as measured by the L-arginine conversion assay, and that this increased NOS activity was calcium dependent (Figuerola and Massmann, 1995). There was no such increase in NOS activity in the endometrium.

The role of a nitric oxide-cGMP system in the human pregnant and non-pregnant uterus was demonstrated when it was shown that cGMP was generated in myometrial strips in the presence of L-arginine or diethylamine/ nitric oxide (a donor of NO), but not L-NAME (Buhimschi et al, 1995). cGMP levels in the myometrium were reduced in samples taken during labour. Inhibitors of NOS increased the contractility of pregnant and non-pregnant uterine muscle strips while diethylamine/nitric oxide inhibited uterine contractility, particularly during pregnancy when compared with non-pregnant and labouring strips (Izumi et al, 1993). Contractility studies of myometrial muscle strips obtained at the time of Caesarean section demonstrated irregular spontaneous contractility which was abolished by L-arginine (Izumi et al, 1993).

These studies (Izumi et al, 1993; Buhimschi et al, 1995) confirmed the earlier animal studies suggesting that NO may be involved in maintaining uterine quiescence during pregnancy and support the observational study which suggested that glyceryl trinitrate patches arrested preterm labour on 20 occasions in 13 women (Lees et al, 1994). However, evidence against the hypothesis that NO may be an endogenous modulator of myometrial tone was presented in a study of 61 patients undergoing elective Caesarean section (Jones and Poston, 1997). Strips of term or pre-term myometrial tissue showed no change in spontaneous contractility when L-arginine, L-NAME or D-MANE was added to the tissue bath. In vivo studies of intrauterine pressure recordings carried out in patients undergoing mid-trimester termination of pregnancy showed no reduction in intrauterine pressure following systemic administration of glyceryl trinitrate, a donor of nitric oxide (Norman et al, 1995). The role of NO in myometrial contractility has yet to be elucidated and so far NO has not been conclusively proven to have a useful clinical application in the prevention of pre-term labour.

### *Vascular effects*

The *in vivo* effects of NO on uterine blood flow were examined when measurements of uterine artery blood flow in non-pregnant sheep were recorded in response to oestradiol 17 $\beta$ , a known vasodilator (Van Buren et al, 1992). Subsequent injection of L-NAME produced significant dose-dependent decreases in uterine blood flow, the authors postulating that L-NAME specifically antagonises the vasodilator effects of oestradiol 17 $\beta$  on the uterine vasculature.

The vasodilator effects of NO on human uterine artery blood flow have been examined *in vitro* studies. A direct effect of endothelial NO formation on human uterine artery relaxation was proposed when it was shown that acetyl choline induced an endothelium-dependent relaxation which was abolished by methylene blue and L-NMMA and reinstated by L-arginine (Jovanovic et al, 1994a). L-arginine was also shown to have a direct relaxant effect on uterine artery rings with or without endothelium, the effect being suppressed by methylene blue and L-NMMA. Addition of a calmodulin inhibitor (known to inhibit constitutive NOS) did not affect the relaxant effects of L-arginine, suggesting that constitutive NOS was not involved (Jovanovic et al, 1994b), while addition of dexamethasone (known to suppress inducible NOS activity) significantly suppressed the relaxation effects of L-arginine. The authors suggested a role for iNOS in the L-arginine-evoked relaxation of the artery. A study using pre-constricted strips of human uterine artery demonstrated a relaxation response when the strips were treated with nicotine (Toda et al, 1994); removal of the endothelium did not influence the response. NOS inhibitors abolished the response, but L-arginine reversed this. Histochemical studies using NOS antiserum demonstrated the presence of immunoreactive fibres in the adventitia, with the authors suggesting that human uterine arteries are innervated by vasodilator nerves which use NO as a neurotransmitter (Toda et al, 1994).



NO may also play a role in foeto-placental blood flow, with NO donors having been shown to relax pre-constricted human placental cotyledons (Myatt et al, 1991). Further studies proved that inhibition of NOS in isolated human placental cotyledons produced increases in perfusion pressure, the authors suggesting that formation of NO contributed to the maintenance of resting vascular tone (Myatt et al, 1992b). NO concentrations, as measured by the Greiss reaction, have been shown to be increased in the foeto-placental circulations of pregnancies complicated by pre-eclampsia (Lyall et al, 1995) and intrauterine growth retardation (Lyall et al, 1996), possibly as a compensatory response to improve placental blood flow.

#### *Neural effects*

NO's production in uterine nerves was suggested with the finding of NADPH diaphorase-positive nerves in the uterus of rats treated with diethylstilbestrol (Shew et al, 1993). NADPH diaphorase is a histochemical stain which reduces a dye in the presence of NADPH but not NAD and neurones staining with NADPH diaphorase have been demonstrated to co-localise with NOS in brain and peripheral tissues (Dawson et al, 1991). Nerve fibres staining positive for NADPH diaphorase were located in rat myometrium, endometrium and adjacent to vasculature, with the authors suggesting that these nerves are capable of NO synthesis and may thus affect myometrial activity (Shew et al, 1993). Further immunohistochemical studies in the rat using a nNOS antibody and a method of retrograde axonal tracing demonstrated that nNOS positive nerves in the uterus can be parasympathetic, originating from the paracervical ganglia, or sensory, originating from thoracic, lumbar and sacral dorsal root ganglia (Papka et al, 1995).

Studies in the human uterus using NADPH diaphorase showed positive axon staining in some, but not all, nerves in the paracervical ganglia, as well as postganglionic



nerves in the myometrium, walls of blood vessels in the parametrium and myometrium, in some cells of endometrial endothelium and the endothelial cells of spiral arterioles (Yoshida et al, 1995). The authors suggested that NO released from the postganglionic nerves may contribute to regulation of uterine vascular resistance by release of NO from nerve endings.

#### Nitric Oxide and Nitric Oxide Synthase in the Endometrium

NOS has been identified in human endometrium using NADPH diaphorase (Yoshida et al, 1995). Subsequent cloning and purification of NOS, and the production of isoform-specific antisera, have enabled further studies to examine the distribution of NOS in endometrium using these more specific markers. Uterine tissue from ten women undergoing hysterectomy for benign disease was demonstrated to contain mRNA for NOS in endometrial glandular epithelium, stroma and myometrium across the cycle (Telfer et al, 1995). NOS-like immunoreactivity, using a monoclonal antibody raised against bovine endothelial NOS, localised to endometrial stroma and blood vessels, with weak staining in glandular epithelium and none in myometrium; this contrasted with NADPH diaphorase activity which was identified in glandular epithelium throughout the cycle, stroma in late proliferative and early secretory samples and in myometrial blood vessels. Subsequent work in 34 hysterectomy specimens using a monoclonal antibody raised against human eNOS and a polyclonal antibody raised against murine iNOS has demonstrated eNOS immunoreactivity to be detected in vascular endothelium and in endometrial glandular cells, with iNOS immunoreactivity localising to glandular epithelial cells (Telfer et al, 1997). Using this antibody there was no stromal staining. There was a variation in the intensity of glandular staining which did not relate to the stage of the cycle. Reverse transcription

polymerase chain reaction confirmed the presence of mRNA for both eNOS and iNOS in gland preparations from proliferative and secretory endometrium.

A study including 26 hysterectomy specimens and three specimens of decidual tissue from early pregnancy examined NADPH diaphorase activity and eNOS and iNOS mRNA content of endometrial glands and stroma across the cycle by Northern blot analysis (Tseng et al, 1996). NADPH staining was localised to endometrial glands, with faint staining in proliferative endometrium but intense staining in late secretory endometrium, suggesting enhanced NOS activity later in the cycle. There was positive staining in endometrial blood vessels. eNOS mRNA was detected in endometrial glands and decidua, with enhanced expression in early and late secretory samples; incubation of glandular cells with relaxin doubled eNOS expression, while incubation with progesterone had no effect. In contrast, iNOS mRNA was detected only in glands from a menstrual endometrium; negative CD45 staining excluded blood contamination and incubation of stromal cells with relaxin or progesterone did not enhance expression. The authors suggested that oestrogen induces eNOS but not iNOS and that release of NO may regulate the onset of menses (Tseng et al, 1996).

These studies (Telfer et al, 1995; Tseng et al, 1996; Telfer et al, 1997) suggest that NOS is expressed in the different compartments of the endometrium, although the effects of cyclical endogenous steroids is unclear. All studies show the localisation of NOS to endometrial blood vessels. Both iNOS and eNOS protein and mRNA are expressed in endometrial glands, with one study showing no variation across the cycle and another showing enhanced expression of eNOS and iNOS mRNA in the late secretory and menstrual phases respectively. The reason for this discrepancy is unclear. Stromal expression of eNOS and iNOS mRNA was absent in two studies (Tseng et al, 1996; Telfer et al, 1997), although the smaller study did suggest stromal expression of NOS mRNA across the cycle (Telfer et al, 1995). Decidualised stroma

expressed eNOS but not iNOS mRNA, although the number of samples was small (Tseng et al, 1996).

Using NADPH diaphorase as a marker for NOS activity, mouse endometrium was examined in early pregnancy and in an artificially induced decidualised endometrium (Moorhead et al, 1995). Positive staining was noted in cells with a distribution identical to macrophages in non pregnant and early pregnancy tissues. As pregnancy progressed, NADPH diaphorase activity increased, paralleling the pattern of decidualization. Positive staining was also present in artificially induced decidual cells. The authors postulated that the function of decidual NO may be to assist endothelial cells in maintenance of vascular smooth muscle relaxation and to inhibit platelet aggregation, thus facilitating blood flow through the placenta (Moorhead et al, 1995).

A number of potential roles for NO in the endometrium may be proposed. NO may play a part in the establishment of early pregnancy. As the developing blastocyst embeds in the endometrium, cells of the syncytiotrophoblast erode the endothelial lining of superficial endometrial blood vessels which have undergone vasodilatation. Maternal blood enters lacunar spaces in the syncytiotrophoblast to establish the uteroplacental circulation and inhibition of platelet activation and adhesion may assist this process. NO's actions as a vasodilator and inhibitor of platelet aggregation may also be important in menstruation, since the initial events in the process include spiral arteriole vasodilatation following a period of vasoconstriction and exposure of subendothelial collagen without the normal platelet adhesion seen in the general circulation (Christiaens et al, 1980).

The precise role of nitric oxide and nitric oxide synthase in the endometrium during the menstrual cycle and in early pregnancy is unclear. The involvement of NO in the

endometrium of patients with menorrhagia has not been addressed. Exogenous steroids have been demonstrated to have an effect on NO production (Yallampai et al, 1994; Figueroa and Massmann, 1995) in animal studies. The effects of high dose exogenous steroids on the expression of endometrial NOS are examined further in chapter five.

## Endothelins and their Receptors

The endothelins (ETs) comprise a family of 21 amino acid peptides, first isolated from the supernatant of porcine endothelial cells. In 1988, Yanagisawa et al demonstrated that this supernatant caused an endothelium-independent slow-onset contraction of isolated porcine coronary artery (Yanagisawa et al, 1988). The vasoconstrictor, known as endothelin, was purified and identified as a 21 amino acid peptide with free amino- and carboxy-termini. Dose-response experiments revealed endothelin to produce a vasoconstrictor response greater than angiotensin II, vasopressin or neuropeptide Y, and in vivo studies in anaesthetised rats showed that the vasoconstrictor response was sustained for 40-60 minutes. This endothelin is now known as endothelin-1 or ET-1.

Unusually, the gene for endothelin was cloned and sequenced before the peptide had been fully pharmacologically characterised and analysis of the human genomic DNA predicted two further 21 amino acid isoforms, ET-2 and ET-3 (Inoue et al, 1989). In humans, the gene for ET-1 is located on chromosome 6, for ET-2 on chromosome 1 and for ET-3 on chromosome 20.

### Synthesis

All endothelins are produced from biologically inactive intermediates. ET-1 is synthesised as a 203 amino acid peptide, pre-proendothelin. Proteolytic cleavage results in the 38 amino acid intermediate, proendothelin-1 (proET-1), and a further proteolytic cleavage mediated by an endothelin converting enzyme (ECE), results in ET-1 (Yanagisawa et al, 1988). Two ECEs have been identified (Xu et al, 1994;

Emoto and Yanagisawa 1995). ET-2 synthesis is thought to occur through synthesis of pre-proET-2 and ET-3 via pre-proET-3. Dense secretory granules are difficult to detect in endothelial cells using transmission electron microscopy, suggesting that endothelins are not stored but are produced on demand by de novo synthesis (Cameron and Davenport, 1992).

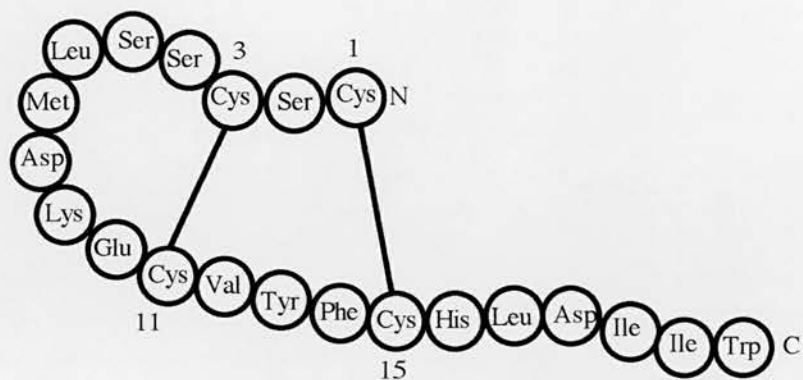
The basic structure of the endothelin molecules is shown in figure 1.2 and bears a strong similarity to the sarafotoxins, a group of peptide toxins from the venom of the burrowing asp *Attractaspis engaddensis*, which cause severe coronary vasospasm in snake-bite victims (Kloog and Sokolovsky, 1989).

#### *Endothelin-1*

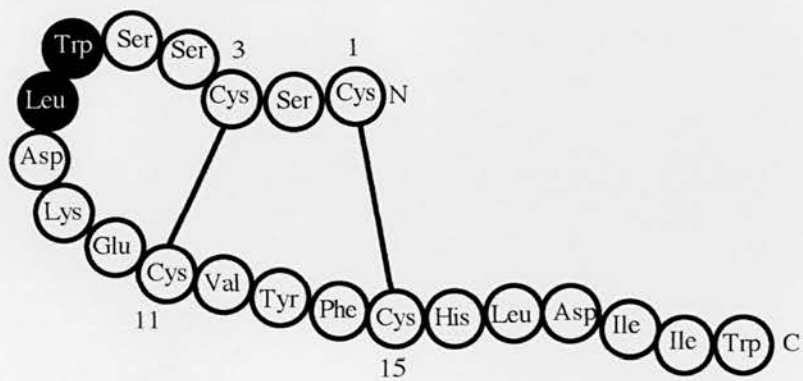
ET-1 has a molecular weight of 2492 Daltons and its 21 amino acids are linked by two disulphide bridges joining amino acids 1 and 15, and 3 and 11. ET-1 has vasoconstrictor effects in almost all animal species. These effects are mediated by an increase in cytosolic calcium, although ET-1 does not appear to act exclusively on calcium channels (Yangisawa et al, 1989). ET-1 has also been demonstrated to release eicosanoids and endothelium derived relaxing factor (nitric oxide) from perfused vascular beds (de Nucci et al, 1988).

In addition to vasoconstrictor and vasopressor properties, a number of non-vascular pharmacological effects have also been identified. ET-1 has been demonstrated to exert a powerful contractile effect on isolated human myometrium taken from the non-pregnant and term pregnant uterus (Word et al, 1992; Wolff et al, 1993) and potentiates the in vitro contractile response of pregnant, but not non-pregnant, myometrial strips to oxytocin (Valenzuela et al, 1995). ET-1 exerts a constrictor effect

Human Endothelin - 1



Human Endothelin - 2



Human Endothelin - 3

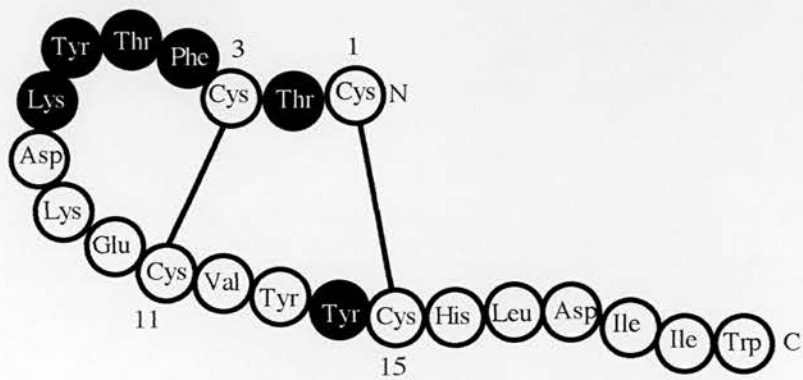


Figure 1.2. The amino acid structures of the mature endothelin isoforms, ET -1, ET -2 and ET -3. The dark circles indicate differences in amino acid structure of ET -2 and ET -3 compared with ET -1.

on smooth muscle in airway and intestine (de Nucci et al, 1988), and has mitogenic and neuroendocrine properties (Haynes and Webb, 1993).

#### *Endothelin-2*

ET-2 has a molecular weight of 2548 Daltons and differs from ET-1 in only two amino acids at positions 6 and 7. In contrast to the body of work on ET-1, there is very little known about the actions of ET-2, however it has been demonstrated to have a more powerful vasoconstrictor action than either ET-1 or ET-3 (Inoue et al, 1989).

#### *Endothelin-3*

ET-3 has a molecular weight of 2644 Daltons and differs from ET-1 in positions 2, 4, 5, 6, 7 and 14. ET-3-like immunoreactivity was initially demonstrated in porcine brain homogenate, suggesting that ET-3 may function as a neuropeptide (Shinmi et al, 1989.). As well as its actions on vascular smooth muscle, it exhibits fibrinolytic activity (Korbut 1989) and acts as an anti-aggregatory peptide (Lidbury et al, 1989).

#### Endothelin Receptors

The identification of the three different isoforms of endothelin led to comparative studies of their efficacy. In vivo studies suggested that, while ET-3 may have only 25-50% of the vasoconstrictor activity of ET-1, it produced a significantly more pronounced transient pressor response than ET-1 (Inoue et al, 1989). This was thought to be due to the different receptors involved, a receptor with a high affinity for ET-1 being responsible for the vasoconstrictor effects and a receptor with a high affinity for ET-3 leading to a transient vasodilatation. Cloning of cDNA for the two



receptor subtypes demonstrated a receptor with a higher affinity for ET-1 than ET-3 localised to vascular smooth muscle and designated ET<sub>A</sub> (Arai et al, 1990), and a receptor with an equal specificity for all 3 subtypes which localised to endothelial cells and designated ET<sub>B</sub> (Sakurai et al, 1990).

Molecular biology studies in rat aortic smooth muscle demonstrated mRNA encoding ET<sub>A</sub> but not ET<sub>B</sub> receptors, and predicted that ET<sub>A</sub> receptors were expressed in vascular smooth muscle (Sakurai et al, 1992). The ET<sub>A</sub> receptor antagonist BQ123 causes a parallel shift to the right of ET-1 induced vasoconstriction in human blood vessels, suggesting that the vasoconstriction response in humans is mediated predominantly via ET<sub>A</sub> receptors (Davenport et al, 1993; Maguire and Davenport, 1993). By contrast, RT-PCR in cultured endothelial cells detected mRNA for ET<sub>B</sub> but not ET<sub>A</sub> (Molenaar et al, 1993), suggesting that ET binding to ET<sub>B</sub> receptors was responsible for the initial transient vasodilatation seen on infusion of ET-1 into animals and humans, possibly via the release of endothelium-derived relaxing factors such as prostacyclin and nitric oxide. This was confirmed in studies of isolated human vessels which show no vasoconstrictor response to infusion of selective antagonists for the ET<sub>B</sub> receptor (Davenport et al, 1993; Maguire and Davenport, 1993).

ET receptor subtypes have also been examined in human myometrium. Subtype selective ligands identified ET<sub>A</sub> as the predominant receptor in the non-pregnant myometrium with a smaller population of ET<sub>B</sub> receptors, and tissue bath experiments demonstrated that ET<sub>A</sub> and not ET<sub>B</sub> mediated myometrial contractility (Bacon et al, 1995; H  luy et al, 1995). Expression of mRNA for ET<sub>A</sub> was increased in the myometrium of pregnant compared with non-pregnant women, and this study also suggested that ET<sub>B</sub> had a role in producing relaxation of the lower uterine segment during pregnancy (Wolff et al, 1996).

## Inactivation

Endothelin is inactivated by the enzyme neutral endopeptidase, a membrane-bound zinc-containing enzyme which degrades several bioactive peptides (Sokolovsky et al, 1990; Vijataraghavan et al, 1990). The enzyme has a widespread distribution in the body (Roques et al, 1993), including the uterus where it has been shown to localise predominantly to endometrial stromal cells (Casey et al, 1991). Enzyme activity correlates with progesterone concentration, increasing early in the luteal phase of the cycle and falling during the pre-menstrual phase (Casey et al, 1991; Head et al, 1993).

The net biological action of ET in a particular cell type probably depends on the balance between peptide synthesis, interaction with receptors and degradation. The metabolism of ET and ET receptors in the endometrium is considered in more detail below.

## Endothelins in the Endometrium

### *Animal studies*

Initial work in rabbits provided evidence of ET expression in the endometrium. ET-like immunoreactivity (ET-IR) was demonstrated in endometrial, but not myometrial cells and the addition of oxytocin and vasopressin increased ET-IR from the endometrial cells, the authors speculating that oxytocin and vasopressin were able to potentiate their actions on the myometrium by production of endometrial ET (Orlando et al, 1990). Subsequent studies localised ET-IR to the endometrial surface epithelium in immature rabbit endometrium (Maggi et al, 1991). The addition of oestradiol, with or without progesterone, resulted in decreased staining in the epithelium and increased staining in the endometrial stromal cells suggesting that ET-IR is under ovarian steroid

control. Binding studies performed on the myometrium of oestrogen-treated rabbits using labelled ET-1 and ET-3 indicated two populations of binding sites present in the myometrium, one selectively binding ET-1 (ET<sub>A</sub> receptors), the other showing an equal affinity for ET-1 and ET-3 (ET<sub>B</sub> receptors) (Maggi et al, 1991). These studies suggest that rabbit endometrium not only produces ET-1 but also contains receptors for ET-1, the distribution of which might be under ovarian steroid control. In addition endometrial ET acts on the myometrium in a paracrine fashion through two different ET receptor subtypes to produce myometrial contractility.

A study in the rat uterus using an antiserum to ET-1 demonstrated binding in the endometrial glandular cells, but none in the stromal cells or in the myometrium (Kajihara et al, 1996). In pregnant, and particularly in post partum tissue, staining was observed in glandular cells and in the myometrium. In situ hybridisation confirmed the presence of prepro ET-1 mRNA in the cytoplasm of postpartum glandular and myometrial cells and Northern blot analysis of myometrial tissue demonstrated the presence of a hybridisation band corresponding to prepro-ET-1 in pregnant but not non-pregnant tissue. The authors proposed both a paracrine and an autocrine role for ET-1 in causing myometrial contractions.

#### *Human studies: differential distribution of ET binding sites*

The first study to identify a role for endothelin in the human uterus was published by Davenport et al in 1991. The authors used quantitative in-vitro receptor autoradiography to localise and compare the anatomical distribution of binding sites for iodinated endothelins (ET-1, ET-2 and ET-3) (Davenport et al, 1991). No difference in binding between the three isoforms was identified. There was a significantly higher density of binding sites in the endometrium compared to myometrium. Within the endometrium, the greatest density of binding was to epithelial glandular cells and

blood vessels, with the greatest density at the endometrial-myometrial junction. There was no significant stromal binding. Because of the small number of tissue samples, changes across the cycle were not examined. It was suggested from this distribution that ET could play a role in the haemostatic control of menstruation as well as in endometrial regeneration. Subsequent immunocytochemical studies (Cameron et al, 1992), using antibodies to the carboxy-terminal heptapeptide of ET-1 which cross-reacts with all three isoforms, supported the autoradiography findings showing ET-IR to be localised to the vascular endothelium in both endometrium and myometrium and to endometrial glandular epithelium. The greatest intensity of staining was at the endometrial-myometrial junction.

*Changes in the distribution of ET binding sites across the cycle.*

Cameron et al also examined changes occurring across the cycle (Cameron et al, 1992). In the eight hysterectomy specimens studied there was some variation in the pattern of ET-IR staining according to the time of the cycle. All glands stained positive in secretory phase tissue but a more heterogeneous pattern of glandular staining was apparent in proliferative endometrium. This suggested that the distribution of ET receptors in human endometrium is under ovarian steroid control. Stromal staining was detected in the basal layer in only one specimen from day seven.

Variation in ET-IR across the cycle was assessed in a larger study using tissue obtained by curettage from 41 women and a polyclonal antibody raised against ET-1 with cross-reactivity against both ET-2 and ET-3 (Salamonsen et al, 1992). In contrast to the other studies (Davenport et al, 1991; Cameron et al, 1992), a low level of stromal staining was noted across the cycle in all specimens. In the proliferative phase of the cycle there was little staining in luminal epithelium and no staining in glandular epithelium, but in secretory phase specimens, staining was strong for both

luminal and glandular epithelium. Because of the nature of the specimens, it was not possible to comment on distribution of ET-IR within the layers of the endometrium.

A more recent study examined endometrial biopsies of 59 normally cycling women taken as controls for patients receiving high doses of exogenous steroids, and used the same antibody to ET-1 on paraffin-wax embedded sections of tissue (Marsh et al, 1995). This study showed a low level of stromal staining across the cycle, with a cyclical variation of ET-IR in luminal and glandular epithelium. Maximal staining occurred during the mid-to late secretory phase and this was followed by a decline in glandular staining during the menstrual phase. The results of these studies suggest that endometrial epithelial ET binding is under the influence of endogenous ovarian steroids, increasing in the secretory phase of the cycle at a time when the endometrium is preparing for implantation or menstruation. This study also examined the distribution of ET-IR in the endometrium of patients with menorrhagia (measured menstrual blood loss > 80ml) and showed a different ET-IR to that of the normal menstrual cycle. ET-IR was less intense in glandular and luminal epithelium than that observed at the same stage of the normal cycle, and less intense than stromal immunostaining, which was of similar intensity to the normal menstrual cycle (Marsh et al, 1996).

#### *Synthesis of endometrial endothelin*

The synthesis of ET in the endometrium was studied using RT-PCR on endometrium obtained from hysterectomy specimens (O'Reilly et al, 1992). Messenger RNA for ET-1, ET-2 and ET-3 was demonstrated in all specimens with no variation across the cycle. Northern blot analysis of mRNA for pre-proET across the cycle in 21 hysterectomy specimens demonstrated that the highest levels were found in tissues obtained during the pre-menstrual (defined as day 26-28) and menstrual (day 1-5)

phases (Economos et al, 1992). This was thought to originate from both endometrial glands and stroma since purified preparations of both cell types secreted immunoreactive ET into the culture medium. The study also demonstrated that treatment of stromal cell preparations with TGF $\beta$  or IL-1 $\alpha$  increased levels of prepro-ET-1 mRNA. Northern blot analysis of pre-proET in a preparation of endometrial tissue demonstrated that levels were higher in the menstrual and proliferative phases than in the ovulatory and secretory phases, and ET-IR was detected in a stromal cell preparation (Kubota et al, 1995). Immunocytochemistry using a polyclonal antibody raised against ET-1 and big ET-1 in 33 hysterectomy specimens demonstrated an increase in expression of both these antibodies in the pre-menstrual and menstrual phases (Ohbuchi et al, 1995).

These studies (Economos et al, 1992; Kubota et al, 1995; Ohbuchi et al, 1995) suggest that synthesis of ET is under endogenous ovarian steroid control. Maximal synthetic activity was consistently noted during menstruation in all the studies. There was some variation in activity immediately before and after the menstrual phase between studies, but in general it was increased during the peri-menstrual period compared with at other times of the cycle.

#### *Endothelin receptor subtypes*

Endometrial synthesis of endothelin is one method of exerting control on its actions. Another control mechanism may operate via the different subtypes of endothelin receptors. RT-PCR was used to identify endothelin receptor subtypes in the endometrium across the cycle (O'Reilly et al, 1992). In menstrual endometrium, ET<sub>B</sub> receptor mRNA was increased relative to ET<sub>A</sub> receptor mRNA. In the proliferative phase, the level of ET<sub>A</sub> receptor mRNA was much greater than that of ET<sub>B</sub> receptor



mRNA, with no detectable ET<sub>B</sub> receptor mRNA in the mid proliferative and early secretory phases. By the mid- to late secretory phases, an increase in ET<sub>B</sub> receptor mRNA was observed. These findings were confirmed using Northern blot analysis when mRNA for both ET receptors was detected in endometrial tissue, with a higher level of ET<sub>A</sub> receptor mRNA in the proliferative phase of the cycle compared to the secretory phase (Kubota et al, 1995). ET<sub>B</sub> receptor mRNA expression increased in the menstrual phase.

More recently, quantitative autoradiography has demonstrated the presence of ET<sub>A</sub> and ET<sub>B</sub> receptors in both endometrium and myometrium across the cycle, and also allowed for localisation of ET receptor subtypes within the endometrium (Collett et al, 1996). Both ET<sub>A</sub> and ET<sub>B</sub> receptors were detected in the myometrium with no demonstrable change in the distribution of receptors in myometrium across the cycle. ET<sub>A</sub> receptors were expressed in stroma throughout the endometrium and showed an increase in density within proliferative endometrium compared with secretory and menstrual endometrium. Endometrial ET<sub>B</sub> receptors were expressed at low density in the proliferative phase with an increased expression in the glands of the basal, but not the functional, endometrium in the secretory phase. The highest density of ET<sub>B</sub> receptors was seen in menstrual phase endometrium where they were present in stromal as well as epithelial cells.

In summary, the results of these studies (O'Reilly et al, 1992; Kubota et al, 1995; Collett et al, 1996) are consistent in the distribution of ET receptors across the cycle. ET<sub>B</sub> has been demonstrated to be the receptor which predominates in the menstrual phase of the cycle, with ET<sub>A</sub> showing a widespread distribution during the proliferative phase. A number of potential roles for endometrial ET in the menstrual and proliferative phases of the cycle may be proposed. ET may exert a mitogenic



effect as part of the endometrial repair process (Kubota et al, 1995; Collett et al, 1996). Endometrial ET may also have an effect on endometrial vasculature producing direct vasoconstriction (Yanagisawa, 1988) or vasodilatation, via release of other vasoactive substances such as prostacyclin or nitric oxide (de Nucci et al, 1988). ET may also be involved in the fibrinolytic activity seen in the uterine cavity during menstruation (Korbut et al, 1989), and in the anti-platelet aggregation seen in endometrial blood vessels at the onset of menses (Lidbury et al, 1989).

#### *Endothelin and endothelin receptors in decidua*

A body of work exists to suggest the involvement of endothelin in human pregnancy. ET-IR has been demonstrated in amniotic fluid in a number of studies. Most studies suggest that levels of ET-IR are increased at term compared with mid-trimester, although there appears to be no further increase with the onset of labour (Nisell et al, 1990; Raboni et al, 1991). Increased ET-IR levels have been shown in the amniotic fluid of women in pre-term labour associated with intra-amniotic infection (Romero et al, 1992).

Endothelin-like immunoreactivity has been demonstrated in vessels of the umbilical cord, with an increase in the level of ET-IR with the onset of breathing following delivery suggesting a role in closure of the umbilical vessels (Nisell et al, 1990; Salamonsen et al, 1992). Increased levels of ET-IR have been demonstrated with intrauterine growth retardation associated with abnormal umbilical artery flow and pre-eclampsia (Ihara et al, 1991; McQueen et al, 1993). All three isoforms of endothelin have been shown to produce vasoconstriction of the foeto-placental circulation (Myatt et al, 1992a). Differential localisation of ET<sub>A</sub> and ET<sub>B</sub> receptors has been demonstrated in normal third trimester placentae as well as placentae from pregnancies complicated by pre-eclampsia and intrauterine growth retardation (Rutherford et al,

1993; Kohnen et al, 1997), suggesting a role for endothelin in the modulation of placental blood flow. ET<sub>A</sub> receptor expression was predominantly localised to smooth muscle cells in the proximal regions of the villous tree, with ET<sub>B</sub> receptor abundant in the periphery and decidua. No differences in the distribution of ET receptors were noted between placentae from normal pregnancies and placentae from pregnancies complicated by pre-eclampsia (Rutherford et al, 1993) or intrauterine growth retardation (Kohnen et al, 1997).

While much of the work summarised above has concentrated on the role of endothelin from the mid-trimester onwards, little work has been carried out investigating the role of endometrial endothelin and endothelin receptors in early pregnancy.

One study examined the synthesis and release of endothelin by human decidual cells by reverse-phase high-pressure liquid chromatography (HPLC) and Northern blot analysis in cultured pregnant decidual tissue from 6-8 weeks gestation (Kubota et al, 1992). ET-IR was detected in the medium of the cultured decidual cells. Reverse-phase HPLC showed that the major component of the ET-IR corresponded to ET-1. Northern blot analysis using human pre-proET-1 as a probe showed the presence of pre-proET-1 in decidual cells. This study also examined the likely ET receptor subtype in decidual cells by competitive binding studies and showed the presence of binding sites with a high affinity for ET-1 and ET-2, the authors suggesting a predominance of ET<sub>A</sub> receptors on human decidual cells.

The effects of decidualisation have also been examined in studies where high levels of exogenous steroids have been given to induce decidual change in the stroma (Kubota et al, 1995; Marsh et al, 1995). Treatment with 0.5mg norgestrel and 0.05mg ethinyl oestradiol was administered to three women who were to undergo hysterectomy (Kubota et al, 1995). Northern blot analysis demonstrated an increased expression of

pre-proET mRNA in post-treatment specimens compared to proliferative and secretory phases of the cycle, and expression of mRNA for both ET<sub>A</sub> and ET<sub>B</sub> was observed in the decidualised endometrium. The localisation of endometrial ET-IR following treatment with high dose progestagens was examined in a study of 107 endometrial biopsies from women receiving subcutaneous levonorgestrel (Norplant®) for between three and 12 months (Marsh et al, 1995). ET-IR was assessed using a polyclonal antibody which cross-reacts with all three ET isoforms. Strongest staining was observed in the stroma, with a low intensity of staining in both glands and luminal epithelium.

These studies (Kubota et al, 1992; Kubota et al, 1995; Marsh et al, 1995) suggest that decidualisation of endometrial stroma is associated with an increase in ET binding, and decidualised stromal cells have been demonstrated to express both ET<sub>A</sub> and ET<sub>B</sub> receptors (Kubota et al, 1995). A number of potential roles for endothelin in early pregnancy may be proposed. Endothelin has a potential role in regulating uterine blood flow. In vitro contractility studies of human uterine arteries and veins demonstrated that endothelin gave a slow and long-lasting contraction at concentrations where conventional vasoconstrictors such as noradrenaline had almost no effect (Fried and Samuelson, 1991) and endothelin may also induce vasodilatation via the release of nitric oxide and prostacyclin (de Nucci et al, 1988). ET is known to be mitogenic for a number of tissues (Hirata et al, 1989; Takuwa et al, 1989; Shichiri et al, 1991) and by its paracrine / autocrine actions may play a role in implantation and the establishment of early pregnancy. In vitro studies have demonstrated that ET-1 modulates the release of renin and prolactin from decidual cells (Chao et al, 1994) and that ET stimulates the production of PGF2 $\alpha$  from decidual cells (Schrey and Hare, 1992). Using the ET<sub>A</sub> receptor antagonist, BQ610, the ET<sub>A</sub> receptor was shown to be coupled to the increased prostaglandin production from first trimester decidual cells

(Seki et al, 1995) and ET has been shown to stimulate DNA synthesis in cultured human endometrial stromal cells (Kubota et al, 1995).

The precise role of endothelin in the endometrium during the menstrual cycle and in early pregnancy is unclear. Endothelin has a potential role in the initiation and control of menstruation, endometrial repair and regeneration and in the establishment of an early pregnancy. Regulation of endothelin may be by alterations in endothelin synthesis or by alterations in receptor expression, both of which have been demonstrated across the cycle and in decidualised stromal cells.

Endothelin may have a role in the pathophysiology of menorrhagia, with reduced ET-IR being demonstrated in the glandular and luminal epithelium of patients with proven menorrhagia (Marsh et al, 1996). Endothelin receptor expression is known to be under ovarian steroid control. The effects of high dose exogenous steroids on the expression of endothelin receptors in the endometrium of patients with proven menorrhagia are examined further in chapter six.



## Clinical Aspects of Menorrhagia

### Introduction

Earlier menarche and fewer pregnancies mean that women in the late 20th century experience more episodes of cyclical bleeding during their lifetime than ever before. A community survey of 521 women showed that 30% rated their menstrual losses as "heavy" or "very heavy" with 22% stating that heavy periods interfered with their life (Gath et al, 1987). Complaints of excessive menstrual loss have a substantial impact on gynaecological services and the number of women consulting their general practitioners for menstrual dysfunction is rising. General practitioners participating in the National Morbidity Survey showed that 31 per 1000 female patients consult their general practitioners each year because of irregular or excessive menstruation, a rise in consultations of 73% over 10 years (RCGP 1986). Abnormal uterine bleeding is one of the commonest reasons for which women are referred to hospital by their general practitioners, with almost three quarters of these referrals due to excessive menstrual blood loss (Coulter et al, 1991).

“But nothing could easily be found that is more remarkable than the monthly flux of women. Contact with it turns new wine sour, crops touched by it become barren, grafts die, seeds in gardens are dried up, the fruit of trees falls off, the bright surface of mirrors in which it is merely reflected is dimmed, the edge of steel and the gleam of ivory are dulled, hives of bees die, even bronze and iron are at once seized by rust, and a horrible smell fills the air; to taste it drives dogs mad and infects their bites with an incurable poison.”

Pliny.

In the 1990's, society's attitude to menstruation may have softened compared to that of Ancient Greece, but many women still find heavy monthly menstrual bleeding an intolerable burden. In very few areas of medicine is a patient's complaint of a symptom enough to put in motion a sequence of investigations and treatments which may culminate in major surgery, but this is the case for patients complaining of menorrhagia and objective corroboration of the complaint is seldom sought.

### The Objective Assessment of Menstrual Blood Loss

Menstrual blood loss has been quantified by a variety of means. The earliest was by weight estimates of soiled sanitary material, first described in a thesis by Prussag in 1912. This technique is simple to perform, but carries with it several disadvantages. The sanitary material may be soiled with other substances such as urine, care must be taken to avoid evaporation and, since the technique gives an estimation of menstrual fluid rather than menstrual blood loss, it may not be an accurate predictor of anaemia. The blood content of menstrual fluid is recognised to vary considerably. In a study of 28 regularly menstruating volunteers, the contribution of blood varied from 1.6% to 82% of total menstrual discharge (Fraser et al, 1985).

Radioisotope methods use  $^{59}\text{Fe}$  or  $^{51}\text{Cr}$  to label red blood cells premenstrually and measure radioisotope activity in the circulation before and after menses. Soiled sanitary material is collected and radioisotope activity in the collection is calculated. Menstrual blood loss can be calculated from this in conjunction with the known isotope activity in the peripheral circulation. One source of inaccuracy is the need to make a complete collection of all menstrual blood lost. Whole body radioactivity has been used to try to avoid the need for collection of soiled sanitary protection, but this method does not allow for blood lost from other sources such as gastrointestinal bleeding and has been shown to be associated with errors when menstrual blood losses lie within the normal range (Price et al, 1964).

Measurement of the iron content of soiled sanitary protection has been used as a measure of menstrual blood loss (Cole et al, 1971), with rapid measurement possible using atomic absorption spectrophotometry. Again, this method relies on the accurate collection of all menstrual blood lost.

Perhaps the best known method of assessment of menstrual blood loss is the alkaline haematin method, first described by Hallberg and Nilsson in 1964 (Hallberg and Nilsson, 1964). Again, this method relies on a full collection of all used sanitary materials. These are soaked in a known volume of 5% sodium hydroxide solution until all coloured spots on the pads or tampons have disappeared. Haemoglobin is converted to alkaline haematin and the reaction takes place irrespective of the age of the blood or the presence of any other proteins or mucins. After 20 hours, an aliquot is removed, filtered, and the optical density is measured at 546nm. This is compared with the optical density of a known volume of the patient's own venous blood diluted in 5% sodium hydroxide using the formula:



$$\text{Menstrual blood loss} = \frac{\text{Optical density menstrual fluid} \times \text{volume of menstrual solution}}{\text{Optical density venous blood} \times \text{volume of venous solution}}$$

This method has the advantage of being cheap and simple to perform, and does not require exposure of the patient to radioactive materials. A recovery of  $96.3\% \pm 0.5$  (mean  $\pm$  SEM) was calculated using this technique (Hallberg and Nilsson, 1964). The importance of a full collection of menstrual blood loss must be emphasised and many studies have recommended that the patient wear a tampon during bathing, urination or defecation to avoid loss of any clots.

A similar technique has been recently described in which a non-ionic detergent replaces sodium hydroxide as the diluent (Gannon et al, 1996). One drawback of all these methods is the need for women to collect their menstrual losses, a task which many women find distasteful. The study by Gannon et al achieved a collection in 89% of women invited to participate, although the authors admit that this level of co-operation may not be achievable outwith a research setting. A pictorial blood loss assessment chart has been devised to avoid the collection of used sanitary material (Higham et al, 1990), but this method may be associated with a high rate of false positive results leading to an over diagnosis of menorrhagia (Deeny and Davis, 1994).

The measurement of menstrual blood loss is time-consuming and requires a high degree of motivation from patients. Without such measurements however, the efficacy of surgical and medical treatments cannot be objectively assessed.

## The Definition of Menorrhagia

Menorrhagia is defined as a menstrual blood loss in excess of 80ml. This definition is derived from two large population studies. The first was carried out in Sweden in 1966 on 476 women aged between 15 and 50 years, who were selected at random as part of a health screening programme and invited to measure their menstrual blood loss by the alkaline haematin method (Hallberg et al, 1966). The second study was in Northumbria in 1971 and measured iron in used sanitary materials from 348 women using atomic absorption spectrophotometry (Cole et al, 1971). Menstrual blood loss in these two studies was shown to have a positive skew distribution with a mean menstrual blood loss of 37.5 - 43.4ml, median of 27.5 - 30ml and losses in excess of 80ml lying above the 95th centile. Hallberg's study also demonstrated that when losses exceeded 80ml, 67% of patients were anaemic, confirming that losses in excess of 80ml should be considered as pathological rather than physiological.

Objective measurement of menstrual blood loss is rarely undertaken outwith a research setting. However patients' perception of menorrhagia has been shown to be inaccurate in several studies. In Hallberg's original population survey, women were asked to rate their menstrual blood loss as heavy, moderate or scanty. 45% of women rating their loss as 'heavy' had a measured menstrual blood loss of less than 40ml and 13% had a loss of less than 20ml (Hallberg et al, 1966). A study of 69 women with a convincing complaint of menorrhagia showed that 62% had measured menstrual blood losses of less than 80ml (Fraser et al, 1984). This suggests that other factors, social and psychological, may lead women to seek treatment for their perceived menorrhagia and these factors may also influence the results of treatment.

## The Aetiology of Menorrhagia

Menorrhagia may occasionally be due to systemic disease such as bleeding diatheses or endocrinopathies. Local causes of heavy menstrual blood loss include uterine fibroids, endometrial polyps, complications of undiagnosed pregnancy, intrauterine or pelvic infection, adenomyosis or endometriosis, copper-containing or inert intrauterine contraceptive devices (IUCDs), and cervical or endometrial malignancy (Cameron, 1989). However in the majority of patients undergoing dilatation and curettage because of menstrual disturbance, no significant intrauterine pathology was identified (MacKenzie and Bibby, 1978). Menorrhagia may be secondary to a disturbance of the hypothalamo-pituitary-ovarian axis. This has been demonstrated in patients complaining of menorrhagia at extremes of reproductive age (Fraser and Baird, 1972; van Look et al, 1977) but for the majority of patients with heavy regular periods, no such disturbance has been demonstrated (Haynes et al, 1977).

Investigation of the patient complaining of menorrhagia is aimed at identifying organic pathology, in particular endometrial malignancy. Such investigation is generally unrewarding, the vast majority of patients having no obvious underlying cause for their menorrhagia, so-called 'dysfunctional uterine bleeding' (DUB), and attention has focused on local factors within the endometrium as a cause of excessive blood loss.

## The Pathophysiology of Dysfunctional Uterine Bleeding

A variety of mechanisms have been proposed in the pathogenesis of DUB. There is extensive evidence that endometrial prostaglandins (PGs) play a role. Endometrial prostaglandin levels increase in the secretory phase of the cycle (Maathuis and Kelly, 1978) and overall levels of PGE<sub>2</sub> and PGF<sub>2α</sub> have been shown to be elevated in

women with proven menorrhagia (Cameron et al, 1987). Furthermore, the ratio of PGE<sub>2</sub> (a vasodilator) to PGF<sub>2α</sub> (a vasoconstrictor) is increased in patients with increased menstrual blood loss (Smith et al, 1981). Endometrial fibrinolysis is also thought to play a role in the aetiology of menorrhagia, with excessive fibrinolytic activity being demonstrated in the endometrium of women with increased menstrual blood losses compared with normal controls (Bonnar et al, 1983). Platelet activating factor has actions as a vasoconstrictor and stimulates platelet aggregation, and may play a role in the paracrine control of the uterine vascular bed. There is evidence that it is synthesised in stromal cells (Alecozay et al, 1991). In addition, a variety of cytokines have been implicated in the process of endometrial repair and angiogenesis, and these may be important in the eventual cessation of menstrual bleeding (for review, see Cameron et al, 1996). The potential roles of endothelin, a 21 amino acid peptide with potent vasoconstrictor properties, and nitric oxide, a readily diffusible free radical species with vasodilator properties, have been discussed earlier in this chapter.

#### The Investigation of the Patient Complaining of Menorrhagia

For years, dilation and curettage (D&C) has been the mainstay investigation for abnormal vaginal bleeding, often with the misconception that the procedure is curative and will lead to the resumption of a normal menstrual cycle. In a study of 22 women with menstrual blood losses in excess of 80ml, the effects of D&C were examined by measuring menstrual blood loss using the alkaline haematin method for several months following the procedure (Haynes et al, 1977). In most patients, there was a reduction in menstrual blood loss in the first month after curettage, but by the second month after the procedure the majority of patients had losses at least equal, if not greater than their pre-curettage menstrual blood loss. The technique is not without complications. The

procedure is usually performed under general anaesthesia, and operative complications include uterine perforation and cervical trauma. In a review of 1029 procedures, the overall complication rate was reported as 1.7%, with four patients requiring laparoscopy or laparotomy and one requiring hysterectomy (MacKenzie and Bibby, 1978). D&C is a blind technique and focal endometrial lesions may be missed. When hysterectomy was performed following dilatation and curettage, it was demonstrated that less than half of the uterine cavity was sampled in more than half of cases (Stock and Kanbour, 1975).

With the development of devices for taking an endometrial biopsy through an undilated cervix, there has been a move away from in-patient D&C towards an out-patient approach (RCOG, 1994). In addition, the realisation that young women with regular menstrual cycles are at low risk of developing endometrial hyperplasia and neoplasia has questioned the need for the use of any invasive endometrial biopsy in such cases (Lewis, 1993). Whilst this has led to a dramatic fall in D&C rates in North America, major changes in practice have not been seen in the England (Coulter et al, 1993) or Scotland (Common Service Agency for the Scottish Health Service, 1994). One reason for this may be that in women for whom sampling of the uterine cavity is indicated, there has been some reluctance to abandon traditional D&C in favour of a less invasive, but similarly blind, biopsy technique which may only sample a small proportion of the endometrium. In a study of 25 patients scheduled for hysterectomy who underwent pre-operative endometrial sampling by a Pipelle device or Vabra aspirator, the percentage of endometrium sampled by the Pipelle device was  $4.2\% \pm 0.92\%$  (mean  $\pm$  SEM) and by Vabra aspirator was  $41.6\% \pm 5.7\%$  (Rodriguez et al, 1993).

The development of fibre optic hysteroscopy to provide a panoramic view of the endometrial cavity and facilitate directed biopsy provides a valid alternative to D&C,

with recommendations that it be more widely used in clinical practice (Lewis, 1993). The availability of narrower gauge instruments increases the scope for more cost-effective assessment as an out patient. In a series of 638 outpatient hysteroscopies only 3% of patients required a general anaesthetic because of cervical stenosis (Mencaglia et al, 1987). The diagnostic accuracy of hysteroscopy compares favourably with both hysterosalpingography and D&C. Hysterosalpingography was able to detect only 62% of the endometrial polyps, 74% of the submucous fibroids and 58% of intrauterine adhesion that were detected with hysteroscopy (Hamou et al, 1985). In a review of 276 cases undergoing both hysteroscopy with directed endometrial biopsies and D&C , 81% of cases had hysteroscopic findings agreeing with those of curettage, 16% had hysteroscopic findings more revealing than curettage and 3% had hysteroscopic findings less revealing than curettage (Gimplerson and Rappold, 1988).

With the increasing use of investigations such as hysteroscopy, the percentage of women diagnosed as having dysfunctional uterine bleeding may fall as more organic pathology is detected; it is important to bear in mind however that findings such as small fibroids and endometrial polyps may be incidental to, and not the cause of, excessive menstrual blood loss. A review of 400 patients presenting for out patient assessment of abnormal uterine bleeding, including 253 referrals due to menorrhagia, is presented in chapter two.



## The Medical Management of Menorrhagia

### Introduction

The traditional surgical treatment for menorrhagia is hysterectomy. 60% of women referred to hospital with menorrhagia had a hysterectomy within five years (Coulter et al, 1991) and menorrhagia makes a significant contribution to the 20% lifetime risk of women in the United Kingdom undergoing the procedure (Vessey et al, 1992). While hysterectomy offers a very effective cure for menorrhagia, it is only suitable for those women who have no further wish to conceive and the procedure represents major surgery with post-operative morbidity rates of 25-47% (Dicker et al, 1982; Dwyer et al, 1993). Conservative endometrial ablation techniques offer an alternative surgical treatment option with significantly reduced post-operative morbidity (Dwyer et al, 1993; Pinion et al, 1994). Long term follow up of these techniques is ongoing (Sculpher et al, 1996) but again they are unsuitable for women wishing to retain their reproductive function.

A number of different medical modes of treatment of dysfunctional uterine bleeding are available, with varying degrees of success. Treatments fall into four main groups as summarised in table 1.1.

Randomised controlled trials which measure menstrual blood loss before and after treatment should be the gold standard to assess the treatment of menorrhagia, since it is only when the objective efficacy of treatment is known that patient satisfaction with treatment can be put into its proper context. There are several points to bear in mind when interpreting the results of clinical trials. One is that, because of other influences



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### Hormonal treatments

- synthetic progestagens
- combined oral contraceptive pill (COCP)
- danazol
- gonadotrophin releasing hormone (GnRH) analogues

Prostaglandin synthetase inhibitors

Inhibitors of fibrinolysis

Reducers of platelet fragility

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Table 1.1. Drugs used in the medical management of menorrhagia.

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on perceived menstrual blood loss and the potential side effects of treatments, effective reduction in MBL may not equate with patient satisfaction with treatment. Another problem with many clinical trials is that patients are only included in the study if their MBL is in excess of 80ml, thus giving no information on the efficacy of treatment for those patients with perceived menorrhagia but normal blood losses. Finally, while many authors publish results showing the percentage reduction in MBL, patients may still be losing in excess of 80ml, so the treatment may hardly be classed as curative.

### Hormonal treatments

The use of synthetic progestagens in the treatment of dysfunctional uterine bleeding will be reviewed in more detail later in this chapter.

### *Combined oral contraceptive pill*

The combined oral contraceptive pill (COCP) is used infrequently for the treatment of menorrhagia, being prescribed by only 11% of a national sample of 518 general practitioners (Coulter et al, 1995). Anecdotally, it has long been recognised that women using the COCP for contraception report reduced menstrual blood loss. This observation has been confirmed objectively in both menorrhagic women and normal controls (Nilsson and Solvell, 1967). The results of a large trial comparing ovulation inhibitors with antifibrinolytic therapy or curettage in patients with proven menorrhagia (Nilsson and Rybo, 1970) showed an overall reduction in MBL from a mean of 158ml to 75ml in the 164 patients randomised to receive the COCP, a reduction of 53%. The influence of organic pathology in the form of uterine fibroids was also examined in this study. The reduction in MBL was significantly lower in patients with fibroids (n=35) who had a percentage reduction of only 25% compared with 59% and 66% for women with 'slightly enlarged' (n=65) and 'normal-sized' (n=62) uteri respectively. Side effects were mild with no thromboembolic complications noted. The age range of patients included in the study was 17-51 years.

Part of the unpopularity of this form of treatment, despite its effectiveness, is the fear of thromboembolic disease in the older age group of women who present with menorrhagia. The appreciation that age alone, in the absence of smoking or other predisposing factors such as previous thromboembolic disease or family history, is not an absolute contraindication to its use should widen its availability to women in the over 35 year age group. The mode of action of inhibitors of ovulation is mainly local, with production of an inactive endometrium, and there may also be a reduction in endometrial prostaglandin synthesis and impaired uterine fibrinolysis.

### *Danazol*

Danazol is a synthetic androgen with antioestrogenic and antiprogestogenic activity; it acts by inhibition of pituitary gonadotrophins. Danazol was first used in the treatment of DUB in 1979 (Chimbira et al, 1979). Eighteen patients with proven menorrhagia received danazol 400mg daily for 12 weeks. MBL fell from  $231 \pm 39\text{ml}$  (mean  $\pm$  SEM) to  $135 \pm 33\text{ml}$  at one month. By three months of treatment, ten out of 18 patients were amenorrhoeic and the remainder had a mean MBL of only 3ml, a reduction in MBL of 96%. Two patients withdrew from the study because of side effects. This danazol regimen had some short term "carry over" effect after discontinuing treatment, with MBL three months following discontinuation still significantly less than pre-treatment values, however the authors accepted that the expense of treatment and side effects limited its use in some patients. Later work by the same author looked at reductions in dosage regimes in an effort to balance efficacy against patient acceptability and found that a regimen of 200mg daily in 16 patients with MBL  $>60\text{ml}$  gave reductions in MBL of 78% with no withdrawals because of side effects (Chimbira et al, 1980).

The main limitation of treatment with danazol is the incidence of androgenic side effects such as weight gain (a statistically significant increase of 4.5kg above pre-treatment values with a regime of 400mg daily), muscle cramp and skin rashes (Chimbira et al, 1980). An attempt to improve patient acceptability by comparing a regimen of decreasing doses of danazol (200 mg daily for the first cycle, reducing to 100 mg daily for the second and 50 mg daily for the third) to a continuous regimen of 200 mg danazol daily or a gestagen (norethindrone 5 mg tds from day 19 to 26 of the cycle) in 57 patients with MBL  $>80\text{ml}$  (Higham and Shaw, 1993) showed that, while both danazol regimens were more effective than the gestagen at reducing MBL, the reducing dose regimen did not improve the incidence of adverse side effects. Over half of the danazol treated patients in this study had MBL  $>80\text{ml}$  after treatment.

In a study comparing danazol 200 mg daily to mefenamic acid, the danazol group of 20 patients with objective menorrhagia showed an overall reduction of 60% in MBL, but three quarters of the patients experienced side effects with 40% of the group declining to continue with treatment because of this (Dockeray et al, 1989). This compared to a reduction in MBL of 20% in the mefenamic acid group but with only one third of patients reporting side effects and 10% refusing to continue with treatment because of side effects. Given these limitations, the main use of danazol therapy has been as second line therapy in patients with previous treatment failures or as short term treatment prior to surgery.

#### *Gonadotrophin releasing hormone analogues*

Gonadotrophin releasing hormone analogues act on receptors in the anterior pituitary gland. When administered continuously, an initial surge in production of the anterior pituitary hormones is followed by down-regulation, resulting in a hypo-oestrogenic state and endometrial atrophy. Bleeding patterns in ovulatory patients who did not complain of menorrhagia showed a reduction in the number of days bleeding and amenorrhoea resulted in about one third of patients (Berqvist et al, 1982). The use of luteinising hormone releasing hormone (LHRH) analogue in a small study of menorrhagic patients demonstrated a reduction in MBL from a mean of 131ml pre treatment to a mean of 11ml after three treatment cycles (Shaw and Fraser, 1984)). Long term treatment is limited by unwanted hypo-oestrogenic effects, in particular bone loss, and its use is mainly confined to short term treatment pre-surgery. The use of LHRH agonist in combination with hormone replacement therapy in 20 patients complaining of menorrhagia showed a reduction in menstrual blood loss from a pre treatment median of 68ml (range 23-397ml) to a median of 17ml at three months with 80% of patients expressing satisfaction with their treatment (Thomas et al, 1991).

### *Prostaglandin synthetase inhibitors*

In terms of randomised controlled trials, more has been published on non-steroidal anti-inflammatory drugs (NSAIDs) than any other medical treatment of menorrhagia. The first uncontrolled report was published in 1976 (Anderson et al, 1976). This report highlighted the convenience of a regimen to be taken only during menses and which would also treat dysmenorrhoea. Non-steroidal anti-inflammatory drugs remain a popular choice, being prescribed by a quarter of general practitioners for the treatment of menorrhagia (Coulter et al, 1995). Most studies use mefenamic acid 500mg tds for five days during menses, although reductions in MBL have also been documented using other PG synthetase inhibitors including diclofenac sodium, flurbiprofen, ibuprofen and meclofenamate sodium.

Reductions in MBL in patients with proven menorrhagia ranged from 22-46% (Fraser et al, 1981; Muggeridge and Elder, 1983; Hall et al, 1987; Dockeray et al, 1989; Cameron et al, 1990; Chamberlain et al, 1991; van Eijkeren et al, 1992; Bonnar and Sheppard, 1996). A proportion of patients did not respond to treatment, with post treatment blood losses still in the menorrhagic range. Some studies included women with MBL <80ml, and still showed reductions in MBL, although of a smaller order (19%) for these patients (Fraser et al, 1981). Reductions of 9-38% were also noted in women using IUCDs; again the larger reductions were seen in patients with MBLs in excess of 80ml (Guillebaud et al, 1978; Rybo et al, 1981). The incidence of side effects reported was generally low, suggesting the treatment would be suitable for long term use. This has been borne out in one long term study which used mefenamic acid 500mg tds during menses for more than one year in 38 women with a subjective complaint of menorrhagia (Fraser, 1983). The study showed that measured MBL was reduced from pre-treatment values of  $66 \pm 5$ ml (mean  $\pm$  SEM) to  $49 \pm 10$ ml at six to nine months and  $43 \pm 5$ ml at 12 to 15 months of treatment, an overall reduction of 24% which was sustained during long term treatment. In addition to measured MBL,

subjective symptoms such as the duration of menses, number of days of pain, number of sanitary towels used and the number of capsules ingested also reduced significantly with time, with only two patients having to discontinue because of gastrointestinal symptoms.

Non-steroidal anti-inflammatory drugs are thought to reduce menstrual blood loss by their action as a prostaglandin synthetase inhibitor, although in vivo studies examining endometrial tissue levels of PGE<sub>2</sub> and PGF<sub>2α</sub> in response to NSAID administration have proved inconclusive (Fraser, 1983; Shapiro and Haning, 1983). Plasma levels of mefenamic acid have been shown to correlate with improvements in MBL (Dockeray et al, 1986). The variable results from tissue studies and from some patients in clinical trials may thus be due to inadequate tissue levels in the uterus.

#### *Inhibitors of fibrinolysis*

Tranexamic acid, an inhibitor of fibrinolysis, is used infrequently as first line therapy in the United Kingdom (Coulter et al, 1995) despite a number of trials which show a reduction in MBL of around 50% in patients with DUB (Milsom et al, 1991; Preston et al, 1995; Bonnar and Sheppard, 1996). In a crossover study with flurbiprofen (Milsom et al, 1991), 15 women with proven menorrhagia showed a reduction in MBL from a mean of 295ml (range 81-701ml) to 155ml (range 36-511ml) after treatment with tranexamic acid 1.5gm tds for three days, then 1gm bd for another four days. This compared to a post treatment MBL of 223ml (range 50-636ml) when treated with flurbiprofen 100mg bd for five days, a reduction in MBL of 44% with tranexamic acid and 21% with flurbiprofen. Reported side effects were minor with no discontinuations because of adverse drug effects. Similar efficacy was reported in a comparative trial with norethisterone, involving 46 women with proven ovulatory menorrhagia (Preston et al, 1995). Twenty five received tranexamic acid 1gm qds for



four days and measured MBL was reduced from 175ml (SD 84ml) to 97ml (SD 89ml), an overall reduction of 45%. This compared to a increase with norethisterone 5mg bd from day 19 to 26 of the cycle of 20%, from 173ml (SD 85ml) to 208ml (SD 135ml). The effect of tranexamic acid is dose dependent, with lower doses of 2gm daily producing reductions in MBL of 36% in anaemic women complaining of menorrhagia (Callender et al, 1970). Tranexamic acid produced subjective improvements of 35-51% in patients with perceived menorrhagia, suggesting that it is also effective in patients with MBL <80ml (Vermylen et al 1968; Nilsson and Solvell, 1967). Patients with menorrhagia in association with IUCD use have also benefited from treatment with antifibrinolytic therapy, with a 56% reduction in MBL demonstrated in 19 women after taking tranexamic acid 1.5gm tds for five days during menses (Ylikorkala and Viinikka, 1983).

Side effects are reported in about one third of patients. These are mainly gastrointestinal and again are dose dependent. One factor limiting the widespread acceptance of antifibrinolytic therapy has been the fear of increased thrombotic activity, highlighted by isolated case reports of intracranial thrombosis and central venous stasis retinopathy (Rydin and Lundberg, 1976; Agnelli et al, 1982). Histochemical studies have shown no evidence of reduced fibrinolysis in superficial vein walls following treatment with tranexamic acid 3-4gm daily on a continuous basis for three weeks (Astedt, 1978) and in Scandinavia, where antifibrinolytics have been used as first line therapy for over 20 years, there has been no increase in the incidence of thromboembolic disease during this time (Rybo, 1991).

#### *Reducers of platelet fragility*

Ethamsylate, which has effects on prostaglandin synthesis as well as direct effects on platelet fragility, has been used in the treatment of menorrhagia for a number of years,



but results from the few clinical trials reported are variable. In a small crossover trial with placebo, ethamsylate 500mg qds from five days before the onset of menses and for ten days thereafter produced a 50% reduction in MBL in nine women complaining of menorrhagia, compared with no change in the placebo group (Harrison and Campbell, 1976). A smaller reduction of 19% was noted when 13 IUCD users complaining of menorrhagia were examined. Side effects were reported in 18 out of 53 ethamsylate cycles, and 17 out of 50 placebo cycles. Another study of IUCD users showed only a 7% reduction in MBL after treatment with ethamsylate 500mg qds during menses (Kasonde and Bonnar, 1975). More recent work examined 44 women with MBL >80ml and showed that ethamsylate 500mg qds during menses reduced MBL by 20% after three months of treatment, although actual values of MBL are not reported and over one fifth of patients originally randomised did not complete treatment (Chamberlain et al, 1991). In a randomised controlled trial with mefenamic acid and tranexamic acid, ethamsylate 500mg qds during menses showed no reduction in menstrual blood loss (Bonnar and Sheppard, 1996).

### Synthetic Progestagens for the Treatment of Menorrhagia

Synthetic progestagens have been used in the treatment of menorrhagia for over 30 years and are the most popular drug prescribed in general practice for the treatment of menorrhagia (Coulter et al, 1995). Their use in the past has been largely empirical and illustrates the importance of objective randomised controlled trials. A further problem with the interpretation of trials using synthetic progestagens is the variety of types, dosages and treatment regimens of progestagens used.

The first report of the use of progestagens in the treatment of menorrhagia was in 13 women with subjective menorrhagia, and used norethisterone in varying doses from

day 15 to 26 of the menstrual cycle (Bishop and de Almeida, 1960). The authors reported a subjective improvement in menstrual blood loss in 34 of 52 cycles treated, and this regimen was universally adopted. It was not until 1987 that short term progestagen therapy was examined objectively, when a non-significant reduction in MBL from 131 to 110ml in six women with ovulatory menorrhagia was observed after treatment with norethisterone 5mg bd from day 16 to 26 of the cycle (Cameron et al, 1987). Further work on a regimen using the same dosage from day 19 to 26 of the cycle in 15 women with ovulatory menorrhagia showed a 20% reduction in MBL from 109 to 92ml, but two thirds of patients thus treated had post treatment MBL >80ml (Cameron et al, 1990). A more recent study using norethisterone 5mg bd from day 19 to 26 of the cycle in 21 women with confirmed ovulatory menorrhagia demonstrated a 20% increase in MBL (Preston et al, 1995).

Increasing the length of treatment to 21 days each cycle has given improved results in one small study involving ten patients with proven ovulatory menorrhagia (Fraser, 1990). Following treatment either with norethisterone 5mg tds or medroxyprogesterone acetate 10mg tds, both from day 5 to 26 of the cycle, MBL fell by 35% from 112 to 73ml. Half of the patients thus treated were still losing >80ml after treatment. Proposed modes of action are the down-regulation of endometrial oestrogen receptors when progestagens are added early in the cycle, or inhibition of ovulation.

Where a shorter duration of progestagen dose has proven to be effective is in that minority of patients with anovulatory menorrhagia, when use of norethisterone 5mg tds or medroxyprogesterone acetate 10mg tds, in a regimen from day 12 to 26 of the cycle gave a 50% reduction in MBL in six patients with confirmed anovulatory menorrhagia, with all but one post treatment MBL being below 80ml (Fraser, 1990).

For the majority of patients who present with ovulatory menorrhagia however, short duration progestagens are ineffective.

While the results of systemic progestagens for the treatment of ovulatory dysfunctional uterine bleeding are variable, the results of use of local intrauterine progestagens are more consistent. Inert or copper-containing intrauterine devices have been shown in clinical trials to increase menstrual blood loss (Guillebaud et al, 1976). Intrauterine devices releasing progesterone or a synthetic progesterone, levonorgestrel, were introduced to improve contraceptive efficacy and reduce side effects. The first such device released 65 $\mu$ g of progesterone in 24 hours over one year (Progestasert® IUCD: Alza Corp., Palo Alto, USA.) and reduced MBL in 12 menorrhagic women from a median of 138 to 49ml at 12 months use (Bergqvist and Rybo, 1983). This represented a reduction of 65%, with all women having MBL within the normal range after treatment, however the device was withdrawn from general use because of an excess risk of ectopic pregnancies. The more recent levonorgestrel intrauterine system (LNG IUS) (Mirena®: Leiras Oy, Turku, Finland) releases 20 $\mu$ gm of levonorgestrel in 24 hours over five years (figure 1.3). Initial studies in 20 patients with MBL >80ml (range 80-381ml) demonstrated a reduction in MBL from a median of 176ml before treatment to 24ml at three months (range 0-145ml) and 5ml at 12 months (range 0-33ml) (Andersson and Rybo, 1990). This represented reductions in MBL of 86% at three months use and 97% at 12 months use. Seven out of the 20 patients were amenorrhoeic at the end of one years use. In a comparative study with flurbiprofen and tranexamic acid, LNG IUS again produced reductions of 82% at three months, 88% at six months and 96% at 12 months of use (Milsom et al, 1991). One feature of local progestagen use is the increase in breakthrough bleeding, which was reported in most women during the first three cycles of treatment (Andersson and Rybo, 1990). Although tolerated by most of the women in the study, two requested removal of the device because of this. One further patient expelled the device. The importance of

counselling patients with regard to abnormal bleeding patterns or amenorrhoea was stressed. The LNG IUS is currently only licensed in the UK for contraception. Possible modes of action of progestagen-releasing devices are a reduction in endometrial prostaglandin synthesis (Cameron et al, 1987), the production of an inactive endometrium (Silverberg et al, 1986) and a reduction in endometrial fibrinolytic activity (Bonnar and Sheppard, 1979).

An additional feature of progestagen-releasing devices is their effect on MBL in patients with normal losses. Both devices have also been shown to reduce MBL, Progestasert® by 50% (Rybo and Bergqvist 1977), Mirena® by 63% (Nilsson, 1977) in patients who did not complain of menorrhagia.

The results of a randomised comparative parallel group study comparing the efficacy and patient acceptability of the levonorgestrel intrauterine system and oral norethisterone for the treatment of idiopathic menorrhagia are presented in chapter three.

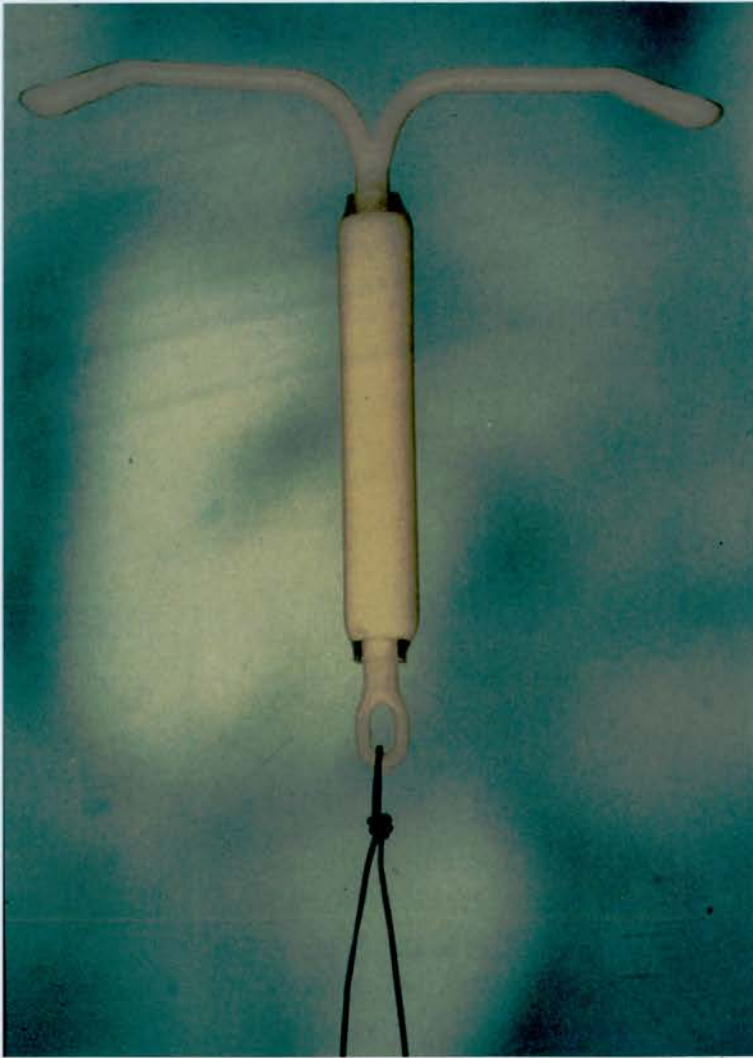


Figure 1.3. The levonorgestrel intrauterine system (LNG IUS). The system consists of a plastic T-shaped frame with a steroid reservoir around its vertical stem. The reservoir contains 52mg levonorgestrel and is covered by a membrane which regulates the release of LNG. The total length of the system is 32mm.

## **Chapter Two**

### **Out Patient Hysteroscopy and Endometrial Biopsy in the Investigation of Abnormal Uterine Bleeding**



Abnormal uterine bleeding is one of the most common reasons for which women are referred to hospital by their general practitioners (RCGP, 1986). Women in the late twentieth century experience more episodes of cyclical bleeding during their lifetime than ever before, an average of over 470 in their reproductive lifetime. Furthermore the widespread use of exogenous steroids for contraception and hormone replacement therapy (HRT) has led to both an increase in the number of women presenting with irregular vaginal bleeding and a prolongation of bleeding cycles beyond the natural menopause.

The development of fibre optic hysteroscopy to visualise the endometrial cavity and facilitate biopsy has altered the approach to the investigation of abnormal uterine bleeding, with a move away from in patient dilatation and curettage (D&C) towards a more cost effective assessment as an out patient.

An out patient hysteroscopy service was established at Glasgow Royal Infirmary in 1989. This chapter comprises an analysis of 400 consecutive patients who attended the clinic between February 1992 and August 1993. Referrals due to menorrhagia accounted for 63%, with a further 17% referred with postmenopausal bleeding and 15% due to abnormal bleeding on HRT. Factors influencing success at accessing the uterine cavity were examined, and the intrauterine pathology identified was reviewed.

### Subjects and methods

Patients were referred from general gynaecology clinics or, in a few cases, direct from their general practitioner. The hysteroscopy clinic was staffed by four gynaecologists, with two nurses and secretarial support. Three of the operators had a minimum of



three years hysteroscopy experience; the fourth had been attending the clinic in a training capacity for six months.

The procedure of hysteroscopy was explained both in a hand-out which patients received before their appointment and verbally by nursing staff at the clinic itself. Pre-medication was offered routinely in the form of 4mg oral diazepam and 75mg intramuscular diclofenac. In the final months of the study period, some women underwent hysteroscopy after an interstitial cervical block using Citanest 3% with Octapressin® (Astra Pharmaceuticals Ltd., Kings Langley, UK) as an alternative to the pre-medication.

The examination was conducted with patients in a modified lithotomy position using a rigid 4mm diagnostic hysteroscope (Olympus Optical Co. Europe, Hamburg, Germany) and carbon dioxide as the distending medium via an automatic hysteroflator (Olympus, Germany). Samples of endometrial tissue were taken using a Pipelle aspirator (Laboratoire CCD, Paris, France) or a Vabra system (Berkeley Medevices Inc., Berkeley, US) and were submitted for pathological assessment and dating using the method described by Noyes et al (Noyes et al, 1950).

### Statistical analysis

Statistical comparison between patient groups was carried out using a chi squared ( $\chi^2$ ) test.

## Results

### *Indication for referral*

Of the 400 referrals, 253 (63%) were referred because of a clinical diagnosis of menorrhagia, that is a subjective complaint of heavy periods. Sixty six (16%) complained of postmenopausal bleeding (PMB), 42 (10%) of unscheduled bleeding on HRT and 17 (4%) of heavy withdrawal bleeds on HRT. Other indications included assessment prior to transcervical endometrial resection (TCRE) (n=5), a history of unopposed oestrogen therapy (n=4), surveillance of a previous endometrial abnormality (n=4), infertility (n=2) and recurrent abortion (n=1).

Of the 253 patients with menorrhagia, 117 complained of heavy menstrual loss with a regular cycle (menstrual interval of  $28 \pm 7$  days), while 136 complained of heavy periods with menstrual irregularity. 84 patients presented with intermenstrual or postcoital bleeding and 85 complained of dysmenorrhoea. Seven patients had of a cycle length of less than 21 days and 16 had a cycle length longer than 35 days.

### *Age distribution*

The youngest patient referred for investigation was 21 years, the oldest 72 years; the median age of referral was 46 years. Looking specifically at the group referred with a complaint of menorrhagia, the youngest was 21 years, the oldest 56 years and the median age of referral was 43 years. Twenty six percent of the women referred with menorrhagia were under 40 years, an age group where the incidence of significant intra-uterine pathology is low (Lewis, 1993). Of the patients referred with postmenopausal bleeding, the age range was 44-72 years, median 54 years. Patients referred because of heavy or unscheduled bleeding on HRT ranged in age from 32-65 years, median 50 years.

### *Factors influencing access to the uterine cavity*

Hysteroscopy was indicated in 332 of the 400 referrals, being omitted at the operator's discretion if he or she felt the procedure would cause unnecessary discomfort while yielding no additional information. The procedure was successful in 260 (78%) patients. In the group under investigation for menorrhagia, the procedure was successful in 185 (84%) of 220 cases.

Endometrial biopsy was attempted in 398 patients, from whom a tissue sample was obtained in 384 (96%) cases. The 14 women from whom endometrial biopsy was not successful were referred for D&C under general anaesthesia, when biopsies were obtained in ten cases.

Factors thought to influence success in accessing the uterine cavity for both hysteroscopy and endometrial biopsy were analysed and are summarised in table 2.1. Of the 400 patients in the study, 30 were nulliparous; a further 29 were parous but had never achieved a vaginal delivery. Twelve gave a history of previous cervical surgery and 66 were postmenopausal with an increased likelihood of cervical stenosis. Some patients had more than one risk factor and were included in more than one group. Operator experience was also analysed, as 116 (29%) of the procedures were carried out by the trainee.

### *Hysteroscopic appearances*

Hysteroscopy was possible in 185 (72%) of the patients referred due to menorrhagia, 29 (44%) of patients presenting with post menopausal bleeding and 32 (70%) of patients complaining of heavy or unscheduled bleeding on HRT. The results are shown in table 2.2. The commonest intrauterine pathologies documented were submucous fibroids and endometrial polyps.

In the group complaining of menorrhagia, the patient's age, cycle and associated symptoms of intermenstrual bleeding were noted to determine whether there was any correlation between such factors and the presence of intra-uterine pathology. The results are shown in table 2.3. There was no statistically significant difference in the presence of intra-uterine pathology between groups.

### *Endometrial histology*

Histological assessment of endometrial biopsies obtained from 366 women in the three main referral groups is shown in table 2.4. In the groups referred because of menorrhagia or abnormal bleeding on HRT, the commonest histological finding was of normal proliferative or secretory endometrium. In the group referred because of PMB, the commonest report was that there was no tissue or that the tissue obtained was insufficient to make a tissue diagnosis.

Over 75% of patients presenting with menorrhagia had no histological abnormality of the endometrium. The endometrial appearances were related to the time in the cycle where known. Eighty three patients were sampled in the first half of the cycle; 78 (94%) showed proliferative endometrium. One hundred patients were sampled in the second half of the cycle; 86 (86%) had secretory changes in the endometrium and 14 (14%) had a proliferative pattern suggestive of anovulatory cycles.

There were two cases of adenocarcinoma detected, both in patients presenting with post menopausal bleeding. Of the four patients with a report of severe atypical hyperplasia, one presented with menorrhagia and three with postmenopausal bleeding; all four patients were over the age of 45. In 68% of patients presenting with postmenopausal bleeding, insufficient tissue was obtained to make a formal histological diagnosis.

### *Complications*

There was one known case of uterine perforation during the series in a patient undergoing investigation for menorrhagia . There were no cases of cervical trauma, excessive bleeding or infection .

### Discussion

Despite the inherent problems of a retrospective analysis, this study does promote an insight into the out patient management of women presenting with abnormal uterine bleeding.

Of the 400 patients studied, menorrhagia was the commonest indication for referral, accounting for almost two thirds of the total. The remaining one third was split almost equally between women presenting with postmenopausal bleeding and those with abnormal bleeding patterns on hormone replacement therapy, a distribution which reflects the increasing numbers of women experiencing artificial bleeding cycles. About half of the group complaining of menorrhagia had an irregular cycle, with 36% also experiencing abnormal bleeding outwith menses.

Where hysteroscopy was attempted, it was successful in 78% of the patients overall and in 84% in the group of the women referred with menorrhagia. As expected, it was significantly more difficult to access the uterine cavity in nulliparous patients compared with patients who had had previous vaginal deliveries. By contrast, parous patients delivered by Caesarean section appeared to present no significant increased difficulty compared to patients with a previous vaginal delivery. Seven patients in whom hysteroscopy was attempted had a past history which might be expected to make investigation more difficult, five of a previous cone biopsy and two of repair

operations with amputation of the cervix. Such a history made hysteroscopy impossible in just over half of cases but the numbers of patients in the cervical surgery group was small. Postmenopausal patients presented significant increased difficulty in passing the hysteroscope, however even then hysteroscopy was achieved in 58% of these patients. The use of local anaesthesia in the form of interstitial or paracervical blocks may have altered success rates but numbers were too small to be analysed separately. This review does not suggest that patients with a history of previous cervical surgery, postmenopausal or nulliparous patients constitute an inappropriate referral for out patient assessment, but that some consideration should be given to more effective local analgesia before attempting the procedure. Operator experience did not appear to make a significant difference to success rates of hysteroscopy in this review. The figures were taken over an eighteen month period during which time a reasonable level of skill had been acquired, emphasising the need for formal supervised training since the skills required for out patient hysteroscopy are very different from those required to do the same procedure under general anaesthesia.

The figures for obtaining an endometrial biopsy show that a biopsy was easier to achieve than hysteroscopy, with access to the uterine cavity or a tissue sample being obtained in 96% of cases. Seventy two patients in the group overall had a failed hysteroscopy and in a further 68 hysteroscopy was not attempted, usually due to patient anxiety; an endometrial biopsy was obtained in 83% and 97% of these patients respectively, suggesting that an initial out patient approach is still valid in most cases. However the limitations of biopsy alone, with regard to blind sampling and area of endometrium sampled should be borne in mind in these groups, particularly when trying to exclude endometrial carcinoma or hyperplasia (Ferry et al, 1993). This is particularly illustrated in the group presenting with postmenopausal bleeding, when 68% of patients had insufficient or no tissue obtained on biopsy to make a tissue diagnosis. While this is suggestive of an atrophic endometrium, there is no way to

ensure that a focal lesion has not missed the sampler unless hysteroscopy is performed in conjunction with endometrial biopsy.

A normal uterine cavity and endometrium was significantly more likely to be reported in patients undergoing investigation for menorrhagia than in the other groups. The hysteroscopic appearances of the uterine cavity showed no abnormality in 73% (135/185) of patients having hysteroscopy for investigation of menorrhagia; this is in keeping with figures generally quoted for the incidence of dysfunctional uterine bleeding. Submucous fibroids were the commonest abnormality, noted in 13% of the menorrhagia group, with endometrial polyps in a further 7.5%, however it is important to bear in mind that such findings may be incidental to rather than the cause of menorrhagia. Fibroids and endometrial polyps were also found in patients complaining of postmenopausal bleeding and abnormal bleeding on HRT, with no significant difference in incidence across the three referral groups. There was one case of endometrial adenocarcinoma observed in a patient presenting with postmenopausal bleeding.

A normal uterine cavity was as likely to be found in a patient with a regular menstrual cycle as an irregular one and in a menorrhagic patient over the age of 40 as under. Fifty seven of the 185 patients having hysteroscopy for investigation of menorrhagia gave a history of intermenstrual bleeding, but this symptom was a poor predictor of intrauterine pathology, with a sensitivity of 42.2% and specificity of 57.8%. There was no significant difference in the finding of intrauterine pathology in patients complaining of abnormal bleeding compared with patients who had no such complaint. It is often thought that patients complaining of intermenstrual bleeding are more likely to have intrauterine pathology. These figures do not support that view.



Endometrial biopsy is performed to exclude endometrial carcinoma or atypical hyperplasia with its potential for malignancy. Seventy three (18%) of the 400 patients referred for endometrial biopsy were under the age of 40 years, rising to 26% in the group referred for investigation of menorrhagia. While this compares favourably to other quoted figures of 45% and 39% (MacKenzie et al, 1978; Coulter et al, 1993), it still represents potential over-investigation, since such patients are at low risk of significant intrauterine pathology (Lewis, 1993). During the study period there were two cases of adenocarcinoma (an overall incidence of 0.5%), both in patients presenting with post menopausal bleeding. There were four cases of atypical endometrial hyperplasia (an overall incidence of 1%). Three of these patients presented with postmenopausal bleeding, one with menorrhagia and an irregular bleeding pattern. All these patients were over the age of 40. These figures confirm the view that endometrial biopsy is an invasive procedure which is unnecessary as a first line investigation in women under the age of 40 complaining of heavy regular periods, and should not be performed unless other risk factors are present (RCOG, 1994).

There was only one known case of uterine perforation during the series in a patient undergoing investigation for menorrhagia . There were no cases of cervical trauma, excessive bleeding or infection. This represents an overall complication rate of 0.25%, which compares favourably to D&C with complication rates as high as 1.7% (MacKenzie and Bibby, 1978).

In conclusion, the results of this study suggest that an out patient approach in the investigation of abnormal vaginal bleeding is valid. There was no sinister intrauterine pathology in any patient under the age of 40 years. In the absence of any other risk factors such as anovulatory cycles, invasive investigation of this group is unnecessary and should not be a first line approach. As expected, the majority of patients presenting with menorrhagia were shown to have dysfunctional uterine bleeding.

Symptoms of intermenstrual bleeding were not predictive of intrauterine pathology in the patients studied. Based on this study these symptoms do not merit first line invasive investigation unless other risk factors are present.

There is an increasing tendency towards day case surgery in the investigation of menorrhagia or other abnormal vaginal bleeding. These figures suggest that for the younger age group of women, no invasive procedure, whether under general or local anaesthetic, is necessary as a first line investigation. Where hysteroscopy and biopsy are required, an out patient approach should be considered, using local anaesthetic techniques to improve hysteroscopic access to the uterine cavity if required since blind out patient biopsy techniques may not adequately sample the endometrium of women at increased risk of hyperplasia or malignancy.

Patient group	Successful hysteroscopy	Successful endometrial biopsy
Previous vaginal delivery	231 / 283 (82%) <sup>a</sup>	331 / 341 (97%) <sup>b</sup>
Nulliparous	12 / 25 (48%) <sup>a</sup>	25 / 29 (86%) <sup>b</sup>
Previous Caesarean section	17 / 24 (71%)	27 / 29 (93%)
Previous cervical surgery	3 / 7 (43%)	7 / 12 (58%) <sup>c</sup>
No previous cervical surgery	257 / 325 (79%)	375 / 388 (97%) <sup>c</sup>
Post menopausal status	29 / 50 (58%) <sup>d</sup>	62 / 66 (94%)
Pre menopausal status	184 / 219 (84%) <sup>d</sup>	247 / 253 (98%)
Trainee hysteroscopist	71 / 98 (72%)	108 / 116 (93%)
Experienced hysteroscopist	187 / 230 (81%)	270 / 276 (98%)

Table 2.1. Success in accessing the uterine cavity.

Factors influencing success in accessing the uterine cavity for hysteroscopy and endometrial biopsy in the different groups. The results are shown as number of patients and the percentage of patients in which hysteroscopy was successfully attempted. Statistical significance ( $\chi^2$  test) is as follows: <sup>a,b,c,d</sup>  $p < 0.05$ .

Appearances	Menorrhagia n=185	PMB n=29	HRT n=32
Normal cavity and endometrium	135 (73%) a,b	7 (24%) a	16 (50%) b
Uterine fibroids	24 (13%)	1 (3%)	6 (19%)
Endometrial polyp	14 (7.5%)	2 (7%)	3 (9%)
Endocervical polyp	2 (1%)	3 (10%)	0
Atrophic endometrium	3 (2%) c,d	15 (51%) c	7 (22%) d
Menstrual endometrium	1 (0.5%)	0	0
Endometrial hyperplasia	0	1 (3%)	1 (3%)
Endometrial adenocarcinoma	0	1 (3%)	0
Other*	8 (4%)	0	1 (3%)

\*Other includes three patients with an IUCD, one with a subseptate uterus, one with adenomyosis, one with a sterile pyometra and two patients in whom the view obtained was inadequate to comment.

Table 2.2. Hysteroscopic appearances.

Hysteroscopic appearance of each of the three main referral groups. PMB=postmenopausal bleeding, HRT= heavy or unscheduled bleeding on hormone replacement therapy. The results are shown as number of patients and the percentage of patients in that referral group. Statistical significance ( $\chi^2$  test) is as follows: a,b,c,d  $p<0.05$ . Some patients had more than one intrauterine pathology noted.

Characteristics	Normal cavity and endometrium	Intrauterine pathology
Regular menstrual cycle (n=94)	71 (76%)	23 (24%)
Irregular menstrual cycle (n=91)	64 (70%)	27 (30%)
Intermenstrual bleeding (n=57)	38 (67%)	19 (33%)
No intermenstrual bleeding (n=115)	89 (77%)	26 (23%)
Age under 40 years (n=46)	38 (83%)	8 (17%)
Age 40 years or over (n=139)	97 (70%)	42 (30%)

Table 2.3. Patient characteristics.

The presence of intrauterine pathology according to cycle regularity, presence of intermenstrual bleeding and patient age. The results are shown as number of patients and the percentage of patients in that group. There was no statistically significant difference in the presence of intra-uterine pathology between groups.

Histology	Menorrhagia (n=248)	PMB (n=61)	HRT (n=57)
Proliferative or secretory endometrium	189 (76%) a,b	4 (6%) a,c	30 (52%) b,c
Simple hyperplasia	8 (3%)	1 (2%)	5 (9%)
Atypical hyperplasia	1 (0.5%)	3 (5%)	0
Endometrial adenocarcinoma	0	2 (3%)	0
Atrophic endometrium	2 (1%) d	10 (16%) d	7 (12%)
Menstrual endometrium	17 (7%)	0	2 (3%)
Endometrial polyp	6 (2%)	0	1 (2%)
Exogenous hormone effects	11 (4%)	0	1 (2%)
Endometrial metaplasia	1 (0.5%)	0	0
Insufficient / no sample	14 (6%) e,g	42 (68%) f,g	12 (20%) e,f

Table 2.4. Endometrial histology.

Endometrial histology obtained in each of the three main referral groups. PMB=postmenopausal bleeding, HRT=heavy or unscheduled bleeding on hormone replacement therapy. Percentage figures show the proportion of cases within the referral group. Statistical significance ( $\chi^2$  test) is as follows: a,b,c,d,e,f  $p < 0.05$ . Some patients had more than one type of histology noted.

## **Chapter Three**

**Randomised Comparative Study of the Levonorgestrel Intrauterine  
System and Norethisterone for the Treatment of Idiopathic Menorrhagia.**



This study was designed to compare the effectiveness of the levonorgestrel intrauterine system (LNG IUS) with norethisterone for the treatment of idiopathic menorrhagia. The norethisterone regimen chosen was not the dose recommended in the British National Formulary, but an increased dose and duration of administration which has been demonstrated to be effective in one small study (Fraser, 1990).

### Subjects and Methods

The study was a randomised comparative parallel group study with patients acting as their own controls in a pre-treatment cycle. For the study to have an 80% power to detect a difference of 45ml between treatments, it was calculated that 22 patients would be required for each treatment arm.

Women complaining of heavy regular periods were recruited into the study from gynaecology outpatient clinics. The study was granted ethical approval from the relevant local ethics committees and patients received a full verbal and written explanation of the study. Entry criteria are shown in table 3.1. Patients were excluded from the study if they had been treated with steroid hormones or anticoagulation agents during the previous three months, or had used injectable hormones for contraception during the previous 12 months.

Patients were asked to collect their menstrual loss over one cycle. The importance of a complete collection was emphasised and verbal and written instructions were given on how to achieve this. Patients were issued with menstrual diaries to complete prospectively, documenting bleeding patterns and menstrual pain during the study.

The degree of menstrual blood loss (MBL) was measured objectively using the alkaline haematin method (Hallberg and Nilsson, 1964) as described in chapter one. The type of sanitary protection used was assessed to make allowances for colour changes caused by dyes in the pads.

Patients with a menstrual blood loss in excess of 80ml proceeded to randomisation by computer generated numbers, the results being sealed in opaque, consecutively numbered envelopes which were opened at the entry visit. The levonorgestrel intrauterine system (Mirena®: Leiras Oy, Turk, Finland) was fitted into the uterus within seven days of the onset of menses. Norethisterone tablets were prescribed at a dose of 5mg three times daily from day 5 to 26 of the cycle over three cycles. A symptom questionnaire was completed and blood was taken to measure haemoglobin and serum ferritin concentrations. Patients were asked to collect their menstrual loss after their first and third treatment cycles. They were reviewed at these times to complete a symptom questionnaire. At the third cycle visit, or at premature termination, haemoglobin and ferritin concentrations were measured again, patients' satisfaction with their treatment was assessed and patients were given the option of continuing with the treatment.

### *Statistical Analysis*

Statistical analyses were carried out both on the per protocol and the intention-to-treat populations. The non-parametric Wilcoxon rank sum test was used to compare the differences between the treatment groups for the primary and secondary outcome measures. If the data were normally distributed the two-sample t-test was used. The time effect within the treatment group was tested by Friedman's two-way ANOVA or by Wilcoxon signed rank test (paired t-test for normally distributed data). Side-effects

were tested with the CATMOD for categorical repeated measures. Calculations were made using SAS® (version 6.07, SAS Institute Inc., Cary, NC, USA).

## Results

One hundred and fifty one patients with a complaint of heavy regular menses were referred by their general practitioner and were asked to collect for an objective measurement of their menstrual blood loss. Of these, 89 completed a collection. Forty eight (54%) had losses within the menorrhagic range ( $> 80\text{ml}$ ), and of these, 44 were willing to proceed to randomisation (figure 3.1).

The characteristics of the 44 patients enrolled in the randomised study are as shown in table 3.2. There was no significant difference between the two groups at baseline.

Of the 44 patients entered in the study, eight withdrew before the end of the three month follow up period, three because of unacceptable drug-related side effects (two in the norethisterone group), two because of perceived treatment failure (both in the norethisterone group), one because of prolonged amenorrhoea (norethisterone group) and one for undefined personal reasons (norethisterone group) (figure 3.1). One patient expelled the levonorgestrel system in the third cycle of treatment. In addition, two patients did not make a final collection of their menstrual loss (both in the norethisterone group). There were no serious adverse events related to either medication.

Table 3.3 shows the effects of treatment on menstrual blood loss. Menstrual blood loss was significantly reduced following treatment compared with baseline for both the levonorgestrel group ( $p < 0.0001$ ) and the norethisterone group ( $p < 0.0001$ ). There

was no difference between the median menstrual blood loss between treatment groups at baseline, but there was a difference between treatment groups at one month and three months ( $p < 0.05$ ). Median menstrual blood loss was reduced to normal in both groups ( $< 80\text{ml}$ ) by the first cycle of treatment.

Individual blood loss changes and individual percentage changes in menstrual blood loss after three months treatment are shown in figure 3.4. Intention-to-treat analysis showed that the levonorgestrel intrauterine device reduced menstrual blood loss by 94% when menstrual blood loss at three months was expressed as a percentage of the control (median reduction 104ml, range -108 to 733ml). When the patient who did not retain the levonorgestrel intrauterine system was excluded from analysis, the group still showed a 94% reduction in menstrual blood loss (median reduction 103ml, range 70 to 733ml). Oral norethisterone reduced menstrual blood loss by 87% (median reduction 94ml, range 56 to 212ml). Six out of nineteen (32%) of patients in the levonorgestrel intrauterine system group and no patients in the norethisterone group were amenorrhoeic following three cycles of treatment.

#### *Haematological parameters*

Ten patients (seven in the LNG group and three in the norethisterone group) who received prophylactic iron treatment during the course of the study were excluded from the assessment of haemoglobin and ferritin concentrations. At baseline, median haemoglobin concentrations were 12.8g/dl (range 11.7-13.8g/dl) in the levonorgestrel group and 13.1g/dl (range 11.1-15.5g/dl) in the norethisterone group. Median ferritin concentrations were 19 ng/l (range 4-49ng/l) in the levonorgestrel group, and 14ng/l (range  $< 1$ -53/l) in the norethisterone group. There was no significant difference in median haemoglobin or ferritin concentrations between treatment groups at baseline.

Following treatment median haemoglobin and ferritin concentrations did not change significantly in either group.

### *Side effects*

Symptoms of intermenstrual bleeding had been reported by 11/22 of the levonorgestrel group and 8/22 of the norethisterone group at baseline, with no significant difference between groups. Following three cycles of treatment as per protocol, 10/19 of the levonorgestrel intrauterine system group reported experiencing intermenstrual bleeding compared with only 2/12 of the norethisterone group. This represents a significant difference in favour of norethisterone ( $p=0.005$ ). Of the 11 patients reporting intermenstrual bleeding prior to treatment with levonorgestrel, five continued to report this symptom following three cycles of treatment. Of the eight patients reporting intermenstrual bleeding prior to treatment with norethisterone, none reported this symptom following three cycles of treatment.

Of the 36 patients who completed treatment, full menstrual diaries were returned by 29 (15 in the levonorgestrel group and 14 in the norethisterone group). There was no significant difference in the median number of days on which mild or moderate-to-severe pain were experienced between treatment groups at three months. When asked about subjective symptoms (headaches, acne, abdominal or back pain, nausea, oedema, weight gain, decreased libido, sweating, hair loss or greasy hair, and increase in body hair), there was no difference in the occurrence of such symptoms between baseline and three months of treatment in either group, and no difference in the occurrence of such symptoms between the norethisterone- and levonorgestrel-treated groups. A small but significant ( $p<0.05$ ) measured weight gain was observed in both groups, 0.6 kg in the norethisterone group and 1.1 kg in the levonorgestrel group, but this was not perceived by the patients. There was a significant decrease in the reported

incidence of mood swings ( $p < 0.05$ ) in both groups from baseline to cycle three, but no difference in the incidence between norethisterone- and levonorgestrel-treated groups. There was a significant decrease in the reported incidence of breast tenderness ( $p < 0.0005$ ) in both groups from baseline to cycle three, with patients receiving levonorgestrel more likely to be reporting this symptom at three months of treatment ( $p < 0.001$ ) than patients receiving norethisterone. In both groups menstruation interfered with the patients' daily life less after three cycles of treatment than at baseline ( $p = 0.001$ ).

#### *Patient satisfaction*

In the levonorgestrel group, 14/22 (64%) women said they liked the treatment 'well' or 'very well' and 17/22 (77%) elected to continue with the treatment at the end of the three months study. In the norethisterone group, 8/18 (44%) reported that they liked the treatment 'well' or 'very well' and 4/18 (22%) elected to continue with the treatment.

## Discussion

This study has demonstrated that both the levonorgestrel intrauterine system, and oral norethisterone at a dose of 5mg three times daily for 21 days of the cycle were highly effective in reducing menstrual blood loss at three months of use (94% and 87% mean reductions in menstrual blood loss respectively). Menstrual blood loss was within normal limits (<80ml) after one month in all patients receiving treatment with the levonorgestrel intrauterine system, and by the end of three months in all but one patient receiving norethisterone. Intermenstrual bleeding was more commonly reported in the levonorgestrel intrauterine system group than in the norethisterone group. Despite this more patients wished to continue with treatment in the levonorgestrel intrauterine system group than the norethisterone group, suggesting that the benefits of reduced menstrual blood loss outweighed the inconvenience of intermenstrual bleeding. Other studies have demonstrated that intermenstrual bleeding decreases with prolonged use of the levonorgestrel intrauterine system (Luukkainen, 1991).

The reductions in menstrual blood loss achieved in this study compare favourably to other studies using first line treatments such as tranexamic acid (Preston et al, 1995; Bonnar and Sheppard, 1996), prostaglandin synthetase inhibitors (Anderson et al, 1976) and the combined oral contraceptive pill (Nilsson and Rybo, 1970), and also to studies using treatments generally considered as short-term options because of side effects such as danazol (Chimbira et al, 1979) and gonadotrophin-releasing hormone analogue (Thomas et al. 1991). The reduction in menstrual blood loss achieved with norethisterone was particularly impressive when compared with previous studies using the drug at different doses and for different durations. Norethisterone is most commonly prescribed for 6 to 10 days in the late luteal phase of the cycle. Such a regimen is effective in regulating an irregular cycle, leading to a predictable withdrawal bleed, but is ineffective for the treatment of ovulatory dysfunctional uterine bleeding.



One report showed a non-significant reduction in menstrual blood loss in six women with objectively proven menorrhagia (Cameron et al, 1987). Another study showed a 20% decrease in menstrual blood loss, but two thirds of patients still had menstrual blood losses in excess of 80ml at the end of treatment (Cameron et al, 1990), while a more recent study demonstrated a 20% increase in menstrual blood loss following treatment with norethisterone (Preston et al, 1995). Such disappointing results have led to suggestions that norethisterone has little or no effect in reducing ovulatory dysfunctional uterine bleeding (Bonnar and Sheppard, 1996). When the dosage and duration of treatment were increased, reductions in menstrual blood loss were improved in a study using norethisterone or medroxyprogesterone acetate from day 5 to 26 in ten women with ovulatory menorrhagia, resulting in a 37% reduction in menstrual blood loss following two cycles of treatment (Fraser, 1990). The present study has shown that, given in high enough doses from early in the cycle, norethisterone is effective at reducing menstrual blood loss, with only one patient still having losses in excess of 80ml after three months treatment. In addition, this regimen has led to a significant reduction in symptoms of intermenstrual bleeding.

The complaint of heavy periods is subjective and this study supports previous observations that half of women who complain of heavy periods have menstrual blood losses within the normal range (Cameron et al, 1990). This has implications for over-treatment, particularly for surgical over-treatment since many perceived 'treatment failures' of medical therapy may represent women whose losses are within the normal range. These women may ultimately seek a permanent surgical cure for their menstrual upset, with the associated morbidity risks.

Both the levonorgestrel intrauterine system and oral norethisterone in this regimen offer an efficacy comparable to the best medical treatment currently available. The levonorgestrel intrauterine system in particular offers a reliable treatment which makes

menstrual blood loss normal in all patients and is well tolerated, with over three quarters of patients wishing to continue with the treatment long term. It has the added advantage of a low incidence of systemic side effects and other studies have demonstrated that it offers highly effective contraceptive cover (Andersson et al, 1994), which is quickly reversible on removal (Andersson et al, 1992). Once fitted, the system frees the patient from the need to take oral medication and it therefore offers an alternative to surgery which until now has been the only real option for long term treatment.

### Conclusions

Both the levonorgestrel intrauterine system and oral norethisterone offer an effective treatment for menorrhagia, but if norethisterone is to be prescribed for women with ovulatory menorrhagia it should be given at a dose of 5mg three times daily from day 5 to 26 of the cycle. The levonorgestrel intrauterine system is associated with higher rates of patient satisfaction and continuation with treatment long-term and offers an alternative to both existing medical and surgical treatments for menorrhagia.

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#### Inclusion Criteria

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- Parous women aged between 18 and 45 years in good general health with a regular menstrual cycle.
  - Normal pelvic examination with a sound measurement of the uterus less than 10 cm and negative cervical cytology.
  - Measured menstrual blood loss in excess of 80 ml.
  - Willingness to accept randomisation to either levonorgestrel intrauterine system or oral norethisterone.
- 

Table 3.1. Entry criteria.

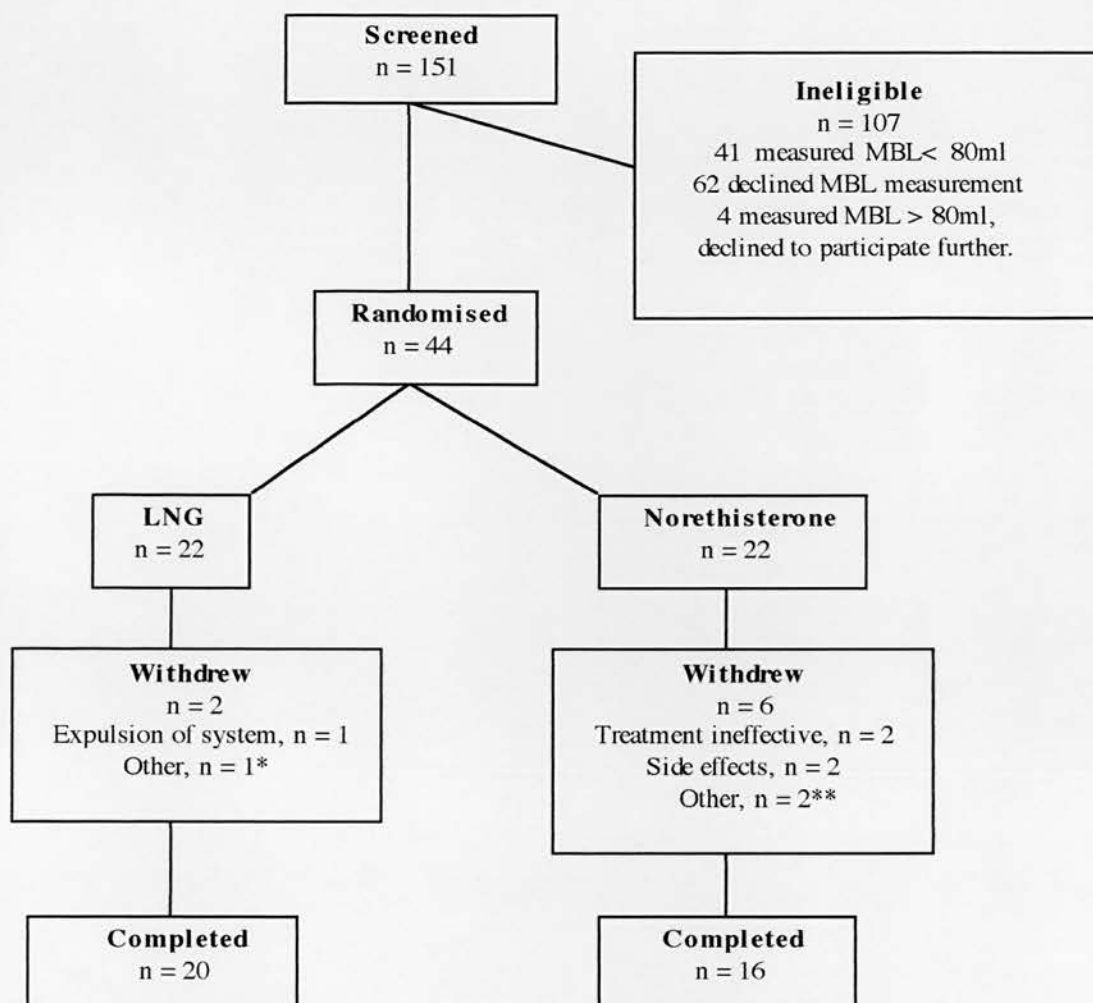


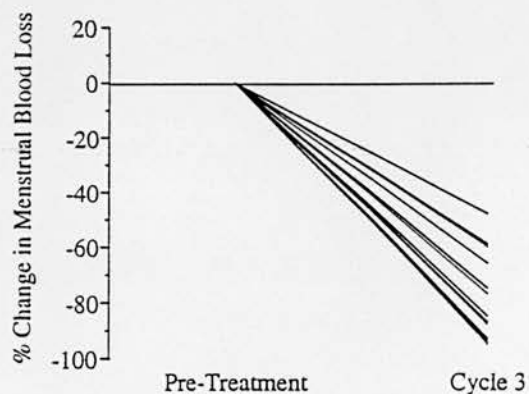
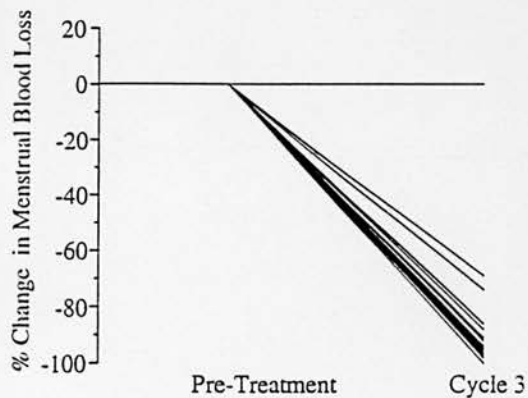
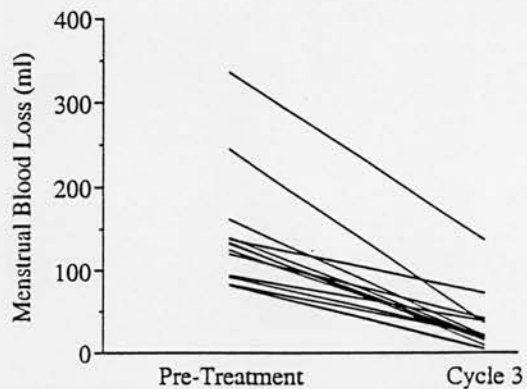
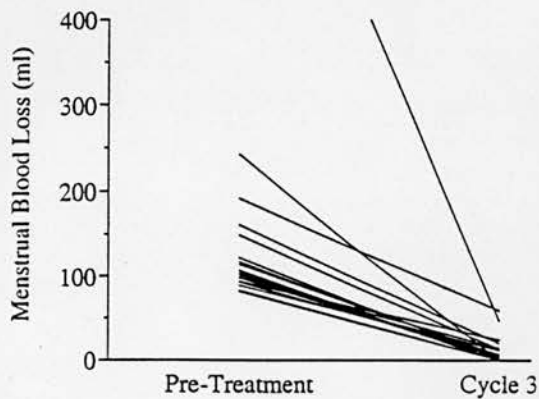
Figure 3.1. Flow chart showing patient progress through the study. \* patient withdrew because of intermenstrual bleeding. \*\* one patient withdrew because of prolonged amenorrhoea, one patient defaulted from the final visit.

	<u>LNG IUS</u>	<u>Norethisterone</u>
	(n=22)	(n=22)
Age (years)	38.5 (31-45)	39 (30-45)
Height (cm)	158.5 (152-170)	159.5 (147-178)
Weight (kg)	69.9 (52.1-116.0)	71.4 (46.4-96.6)
BP    systolic (mm Hg)	121 (100-155)	119 (100-150)
diastolic (mm Hg)	80 (65-95)	79 (64-108)
Parity	2 (1-5)	2 (1-5)
MBL pre treatment (ml)	105 (82-780)	120 (82-336)

Table 3.2. Characteristics of the 44 women enrolled to the study. Data are presented as median (range). LNG IUS = levonorgestrel intrauterine system.

	<u>LNG IUS</u>	<u>Norethisterone</u>
<u>Control cycle (ml)</u>		
Median (range)	105 (82-780)	120 (82-336)
<u>1st treatment cycle (ml)</u>		
Median (range)	16 <sup>a</sup> (0-62);	46 <sup>a</sup> (0-213);
Mean difference (ml)	149	64
95% confidence intervals (ml)	-159 to 458	-85 to 214
<u>3rd treatment cycle (ml)</u>		
Median (range)	6 <sup>b</sup> (0-284);	20 <sup>b</sup> (4-137);
Mean difference (ml)	133	108
95% confidence intervals (ml)	-163 to 429	13 to 203

Table 3.3. Menstrual blood loss measurements in the two groups of women in control and treatment cycles, analysed in the intention to treat population. <sup>a, b</sup>  $p < 0.05$ . LNG IUS = levonorgestrel intrauterine system.



Levonorgestrel  
Intrauterine System

Norethisterone

Figure 3.4. Individual blood loss reductions for norethisterone and LNG IUS. The upper two figures show the menstrual blood loss before and after three cycles of treatment. The lower two figures show percentage change in MBL in cycle three treatment compared with pre-treatment cycles. LNG IUS=levonorgestrel intrauterine system.



## Chapter Four

Epithelial Cell Morphometry, Scanning Electron Microscopy Assessment,  
and  $\alpha 2$  laminin and *Dolichos Biflorus* Immunohistochemistry of the  
Endometrium of Patients with Proven Menorrhagia, Before and After  
Treatment with Exogenous Progestagens

The endometrium undergoes profound structural changes during the normal menstrual cycle and following exposure to exogenous progestagens (chapter one). High dose exogenous progestagens have been demonstrated to reduce menstrual blood loss (chapter three). This chapter investigates some of the changes occurring in the endometrium of patients with proven menorrhagia in response to systemic norethisterone and intrauterine levonorgestrel and examines four aspects, changes in epithelial cell height, epithelial surface morphology, epithelial secretory activity and stromal decidualisation.

Epithelial cell height was assessed by morphometric measurements (part one).

Epithelial surface morphometry was assessed by scanning electron microscopy (part two).

Epithelial secretory activity was assessed using *Dolichos biflorus* agglutinin immunohistochemistry (part three).

Stromal decidualisation was assessed using  $\alpha 2$  laminin immunohistochemistry (part four).

Part One. Endometrial Epithelial Cell Morphometry in the Endometrium of Patients  
with Proven Menorrhagia, Before and After Treatment with Oral and Intrauterine  
Progestagens

Materials and Methods

*Collection of endometrial biopsies*

Endometrial tissue was obtained from 23 healthy parous women taking part in the clinical trial described in chapter three. The tissue was collected using a Z-sampler (ZSI, Chatsworth, USA.) in the mid luteal phase of the cycle (median day 21, range day 16-25), was snap-frozen in liquid nitrogen and stored at -70°C until required. The timing of biopsy collection is shown in table 4.1.

Twelve women received intrauterine progestagens via a levonorgestrel intrauterine system (LNG IUS) releasing 20 µg levonorgestrel in 24 hours (Mirena®: Leiras Oy, Turku, Finland), fitted within seven days of the onset of menses. Eleven women received oral progestagens in the form of norethisterone 5mg three times daily from day 5-26 of their cycle.

A second endometrial biopsy was taken in the mid-luteal phase of the first treatment cycle and was snap-frozen and stored as before. The median exposure time of the tissue to intrauterine levonorgestrel was 18 days (range 14-22 days), and to oral norethisterone was 18 days (range 14-20 days). The timing of biopsy collection is shown in table 4.1.

10µm cryostat sections of tissue were cut, mounted onto 3-aminopropyltriethoxysilane coated slides (Sigma, Poole, UK) and air dried. Tissue sections were stained with Harris's haematoxylin (Sigma, UK) and eosin (BDH, Glasgow, UK), dehydrated in a graduated ethanol-xylene series (70%, 90%, 100% ethanol, xylene twice) and mounted in DPX-mounting medium (BDH, UK).

Morphometric analysis of cell height was carried out on endometrial glandular and surface epithelial cells by one observer. Nine arbitrarily chosen sites were measured in areas of biopsy with good epithelial preservation and no stromal disruption, and the median calculated. A bright light field microscope (Orthoplan, Leitz: Leica Ltd., Milton Keynes, UK) with objectives x 63 and wide-angle eyepiece x 12.5 was used with an eyepiece graticule (Leica Ltd., UK). Perpendicular measurements of cell height were made from the apical surface to the basement membrane of the cell (figures 4.1 and 4.2). All measurements were made by one observer.

### Statistical analysis

An unpaired t-test was used to compare exposure time to treatment in the two groups, and epithelial cell height pre- and post-treatment in the two groups. A Wilcoxon signed rank test was used to compare measurements following treatment in the two groups.

### Results

There was no significant difference in exposure time of the tissue to treatment in the two groups.

One patient who had been randomised to receive levonorgestrel was noted to have a proliferative pattern of endometrium in her pre-treatment biopsy. This was assumed to be secondary to anovulation and epithelial cell height measurements were omitted from analysis. Because of tissue disruption, measurements of glandular epithelial cell height were not possible in a further two pre-treatment biopsies and in three post treatment biopsies (one LNG IUS, two norethisterone). Likewise, measurements of surface epithelial cell height were not made in two pre-treatment biopsies and in nine post treatment biopsies (five LNG IUS, four norethisterone).

There was no correlation between glandular or surface epithelial cell height and the timing of the pre-treatment endometrial biopsy (coefficient of correlation 0.16 and 0.10 respectively).

Epithelial cell height measurements are summarised in tables 4.2 and 4.3. There was no difference in glandular ( $p=0.266$ ) and surface ( $p=0.894$ ) epithelial cell height between the two groups before treatment. There was no difference in glandular ( $p=0.294$ ) and surface ( $p=0.054$ ) epithelial cell height between the two groups following treatment.

Following treatment with levonorgestrel, there was a significant reduction in both glandular ( $p<0.001$ ) and surface ( $p<0.01$ ) epithelial cell height.

Following treatment with norethisterone, there was a significant reduction in both glandular ( $p<0.001$ ) and surface ( $p<0.05$ ) epithelial cell height.

Figure 4.1. Photomicrograph of haemotoxylin and eosin staining in a pre-treatment biopsy taken of day 24 of the cycle. Two glands (G) can be seen amid stromal cells (S). A perpendicular measurement of epithelial cell height is taken from the apical surface to the basement membrane as shown. Scale bar represents 50 $\mu$ m.

Figure 4.2. Photomicrograph of haemotoxylin and eosin staining in a post-treatment biopsy taken after 18 days exposure to norethisterone. Three glands (G) can be seen amid stromal cells (S). In contrast to figure 4.1, the glands are lined with a more cuboidal epithelium and the perpendicular measurement of epithelial cell height is much reduced. Scale bar represents 50 $\mu$ m.



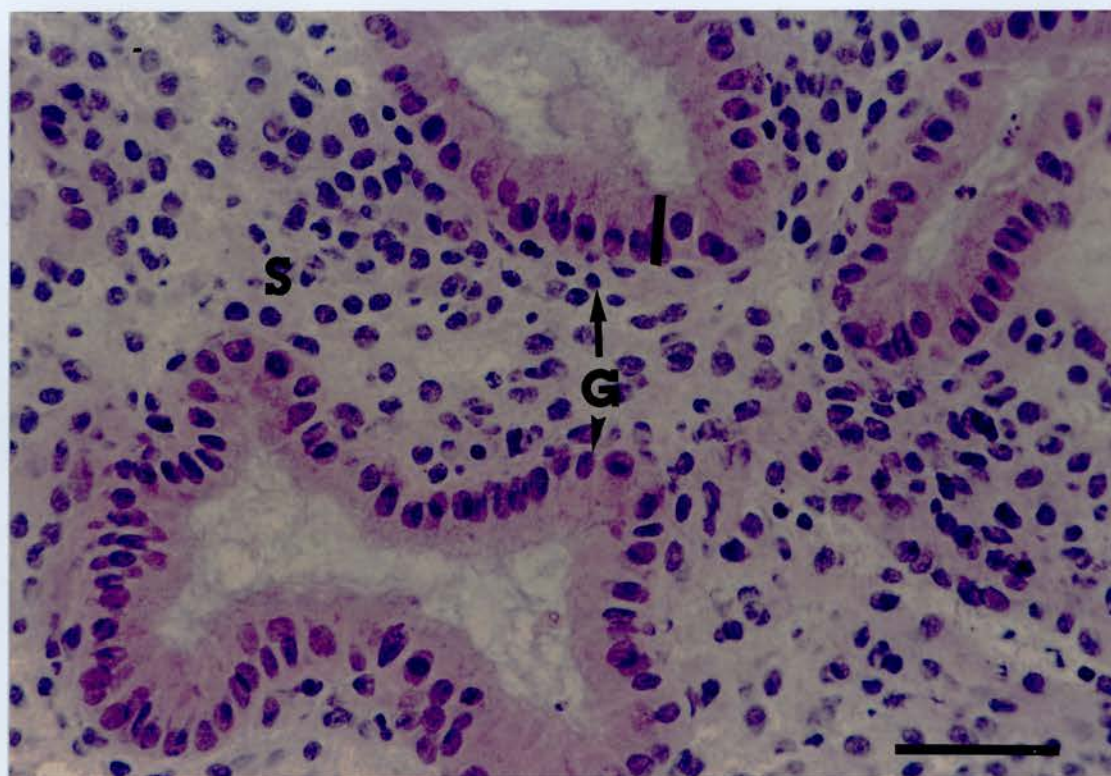


Figure 4.1.

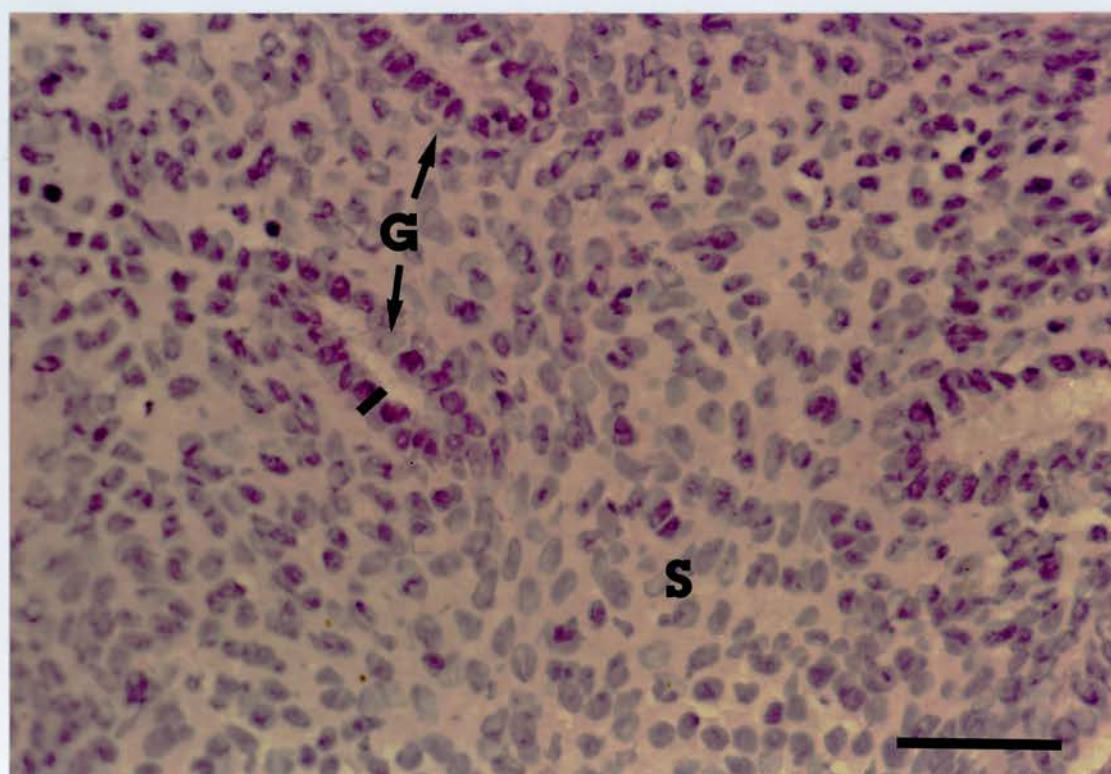


Figure 4.2.



Day of pre-treatment biopsy	Number of patients	Days exposure to progestagens	Number of patients
day 15	-	13	-
day 16	x	14	xx
day 17	x	15	xx
day 18	x	16	xx
day 19	xxx	17	xxxx
day 20	x	18	xxxx
day 21	xxxxx	19	xx
day 22	xxxx	20	xxxx
day 23	xxx	21	xx
day 24	xx	22	x
day 25	xx	23	-
day 26	-	24	-
Total	23	Total	23

Pre-treatment biopsies

Treatment biopsies

Table 4.1. Timing of endometrial biopsies in pre-treatment and treatment cycles. Pre-treatment biopsies are expressed as number of days from last menstrual period. Treatment biopsies are expressed as number of days exposure to exogenous progestagens. x=one patient.

Biopsy	Glandular epithelium		Surface epithelium	
	Pre-treatment ( $\mu$ )	Post treatment ( $\mu$ )	Pre-treatment ( $\mu$ )	Post treatment ( $\mu$ )
1	31 (27-33)	15 (12-16)	23 (19-25)	16 (15-17)
2	35 (31-39)	16 (12-17)	23 (21-29)	16 (14-19)
3	P.M	19 (17-21)	14 (13-15)	P.M
4	33 (29-35)	P.M	23 (21-29)	P.M
5	29 (25-35)	16 (14-17)	27 (25-27)	12 (10-14)
6	Prolif	17 (12-21)	Prolif	12 (10-15)
7	31 (29-33)	14 (8-16)	29 (23-29)	19 (19-21)
8	27 (23-31)	16 (16-19)	31 (29-33)	P.M
9	31 (29-33)	27 (27-29)	23 (19-25)	17 (17-19)
10	33 (33-37)	16 (16-17)	29 (27-29)	P.M
11	27 (23-29)	25 (23-27)	14 (7-17)	P.M
12	27 (25-33)	19 (17-21)	29 (25-29)	19 (17-21)

Table 4.2. Epithelial cell height measurements pre- and post treatment with the levonorgestrel intrauterine system, expressed as median (range). Prolif = proliferative phase endometrium suggestive of anovulatory cycle. P.M = poor morphology. There is a marked difference in median cell height measurements pre- and post-treatment, with little overlap of the ranges.

Biopsy	Glandular epithelium		Surface epithelium	
	Pre-treatment ( $\mu$ )	Post treatment ( $\mu$ )	Pre-treatment ( $\mu$ )	Post treatment ( $\mu$ )
1	31 (29-37)	19 (16-21)	21 (17-21)	17 (15-19)
2	31 (29-35)	13 (12-13)	19 (17-21)	P.M
3	25 (21-27)	19 (19-21)	33 (29-35)	P.M
4	37 (31-41)	19 (15-19)	33 (33-39)	25 (21-25)
5	35 (31-37)	16 (13-17)	29 (27-31)	12 (9-13)
6	25 (25-31)	21 (19-21)	23 (21-25)	19 (17-23)
7	17 (15-21)	P.M	14 (13-16)	P.M
8	16 (10-19)	14 (12-16)	16 (14-21)	14 (12-14)
9	P.M	P.M	P.M	P.M
10	39 (35-41)	17 (16-21)	P.M	19 (16-19)
11	16 (13-17)	17 (14-19)	17 (13-27)	14 (12-19)

Table 4.3. Epithelial cell height measurements pre- and post treatment with norethisterone, expressed as median (range). P.M = poor morphology. In contrast to the levonorgestrel-treated group there is a less marked difference in median cell height measurements pre- and post-treatment, particularly with regard to surface epithelial cell height, and the ranges are not so distinctly separate.

Part Two. A Scanning Electron Microscopy Assessment of Endometrial Surface Epithelium of Patients with Proven Menorrhagia, Before and After Treatment with Oral and Intrauterine Progestagens.

Materials and Methods

*Collection of endometrial biopsies*

Seventeen endometrial biopsies were obtained from 15 healthy parous women with proven menorrhagia taking part in the clinical trial described in chapter three. The tissue was collected as described in part one of this chapter in the mid luteal phase of a control cycle (median day 21, range day 19-24) (n=8), and in the mid-luteal phase of the first cycle of treatment with either oral norethisterone (n=3) or with intrauterine levonorgestrel (n= 6). Two patients had both a control and a treatment biopsy taken. The median exposure time of the endometrium to the treatment was 16 days in the norethisterone group (range 15-20 days) and 20 days in the levonorgestrel group (range 14-21 days).

The samples were rinsed briefly in 0.9% saline solution, fixed in glutaraldehyde 2.5% in cacodylate buffer 0.1M for at least 24 hours and then were processed according to the scanning electron microscopy protocol of the Tennent Institute of Ophthalmology, Western Infirmary, Glasgow. Briefly the tissue was rinsed four times in cacodylate buffer (BDH, UK) over 60 minutes at room temperature, fixed in 1% osmium tetroxide (Oxkem Ltd., Holton, UK) for 60 minutes, rinsed again four times in cacodylate buffer over 60 minutes and dehydrated in a graduated ethanol series ( 25%, 50%, 75%, 100% x four), each step in the series taking 15 minutes. The samples were then subjected to critical point drying with carbon dioxide followed by sputter coating with gold in a high-vacuum evaporator to achieve a coating thickness of 4 nm.

The specimens were stored in a dessicator containing silica gel until required. The microscope used was a JSM 6400 scanning electron microscope (JEOL, Tokyo, Japan) and a blind assessment of the specimens was made by one observer. The specimens were studied at different angles and tilts at an accelerating voltage of 10 kV. Photographs were taken using Kodak TMAX 100 film 120 format or Ilford FP4 plus, 120 format.

Preliminary studies on random endometrial biopsies showed that the processing resulted in 'worms' of tissue 10-20mm long and 1-2mm diameter. Much of the external surface of some specimens consisted of interlocking fibres with occasional enmeshed red blood cells, presumably a reflection of tissue disruption during the sampling. Detailed surface anatomy could be visualised in gaps in this fibrin network. To expose the internal detail, the specimens were mechanically disrupted by gentle pressure after critical point drying but before gold sputter coating.

## Results

### *Control biopsies*

The surface epithelium was slightly folded and interrupted only by the openings of the endometrial glands at distances of approximately 150 $\mu$ m from each other (figure 4.3). At higher magnifications (figures 4.4), the surface epithelium was observed to consist of polygonal cells of about 2.5 $\mu$ m diameter, some bearing dense tufts of microvilli on their apical surface, others with cilia projecting from the surface.

### *Treatment biopsies*

While most biopsies exhibited the features described below, others were of a more heterogeneous appearance. Within the same sample, some areas were similar to that

seen in the control biopsies, with cilia and microvilli on the apical surface of the cells, but other areas of the same biopsy showed the features described below.

1. Defective ciliogenesis.

Cilia were absent and the tufts of microvilli appeared shorter and sparser. This was a consistent finding in all the levonorgestrel-treated biopsies and in all but one of the norethisterone-treated biopsies (figures 4.5 and 4.6).

2. Collapsed cells.

In some of the post treatment biopsies, the apical surface of the cells had a collapsed appearance (figure 4.7). This feature was associated with the levonorgestrel-treated biopsies.

3. Flattened cobblestone epithelium.

The surface epithelium was devoid of any features with the cell margins showing clearly (figures 4.8, 4.9 and 4.10) . This feature was more pronounced with the levonorgestrel-treated biopsies.

On the basis of these three features, the control biopsies and the post-treatment biopsies could be differentiated with a sensitivity of 0.84, specificity of 0.96 and positive predictive value of 0.91.

On the basis of the appearances of collapsed cells and a pronounced cobblestone epithelial pattern, the levonorgestrel-treated biopsies could be differentiated from the norethisterone-treated biopsies with a sensitivity of 0.76, specificity of 0.83 and a positive predictive value of 0.91.

Figure 4.3 (x1000). Control biopsy.

View over surface epithelium between two gland openings at day 19. The ciliated (C) and microvillous (V) cells typical of this stage of the cycle are visible. Scale bar represents 10 $\mu$ m.

Figure 4.4 (x6000). Control biopsy.

Same specimen at higher magnification showing the two different apical cell appearances. Two ciliated cells (C) can be seen amid the microvilli (V) cells. Scale bar represents 1 $\mu$ m.





Figure 4.3.

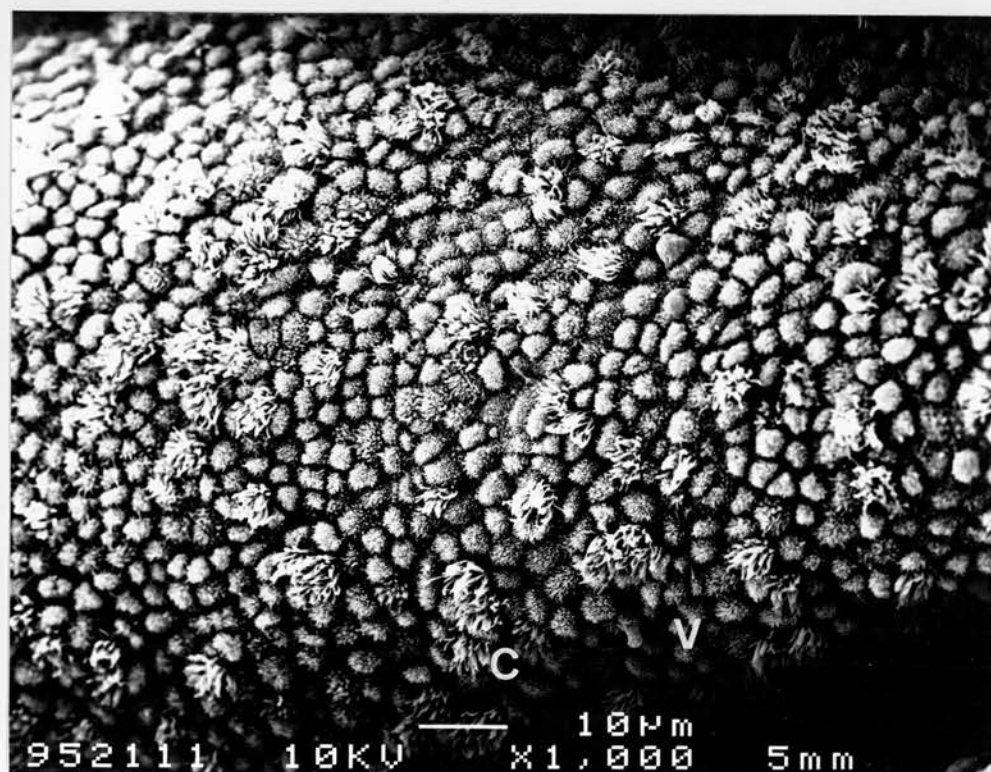


Figure 4.4.

Figure 4.5 (x950). Norethisterone-treated biopsy.

General view over the surface epithelium following 16 days exposure to norethisterone. The opening of the gland (G) is visible on the left. In contrast to the control biopsies, fewer ciliated cells are apparent and the cilia are less well defined.

Scale bar represents 10 $\mu$ m.

Figure 4.6 (x2200). Norethisterone-treated biopsy.

Same specimen at higher magnification showing the surface epithelium between two gland openings. Ciliated cells are absent. Scale bar represents 10 $\mu$ m.

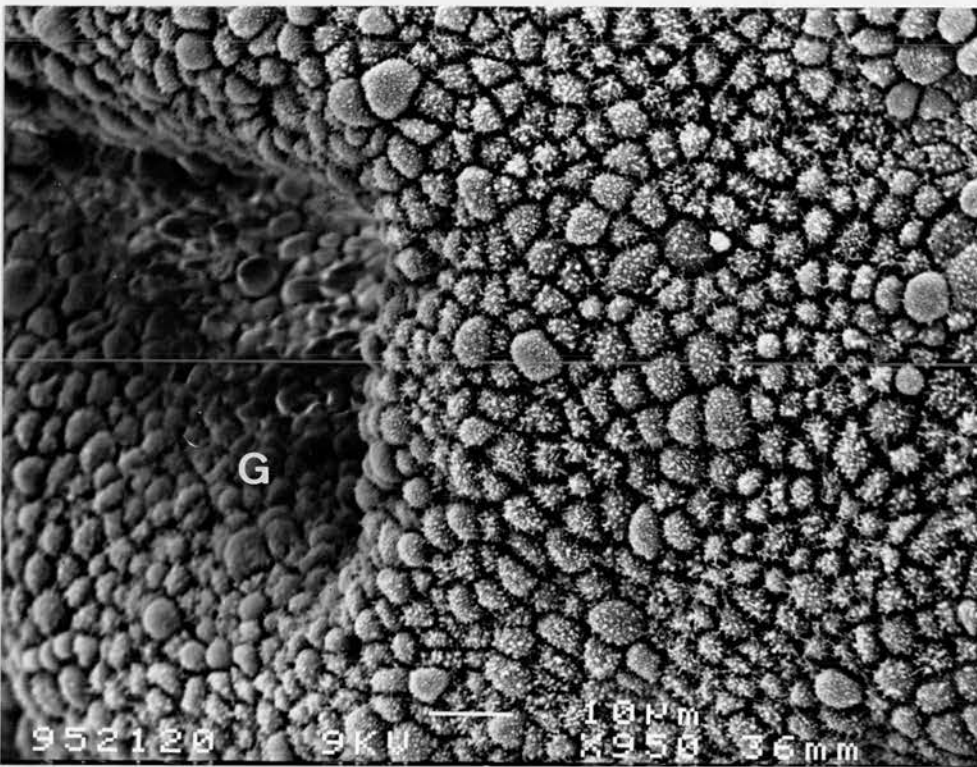


Figure 4.5.

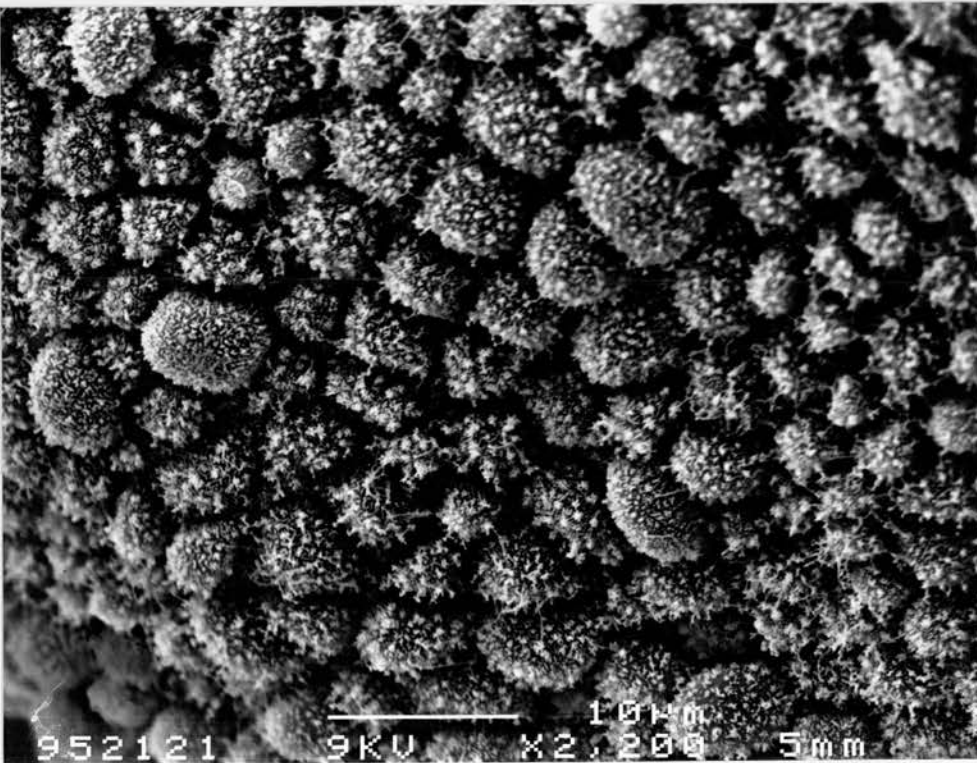


Figure 4.6.

Figure 4.7 (x600). Levonorgestrel-treated biopsy.

General view over the surface epithelium following 19 days exposure to levonorgestrel. To the right is an area disrupted by the sampling and covered by a mesh of fibrin (F). The opening of a gland (G) can be seen at the bottom of the picture, with a second at the top left of the picture. The epithelium between the glands shows a gradation between an area of defective ciliogenesis (D) to an area with a flattened cobblestone (C) appearance to the epithelium. Scale bar represents 10 $\mu$ m.

Figure 4.8 (x2200). Levonorgestrel-treated biopsy.

Surface epithelium between two glands following 20 days exposure to levonorgestrel, showing an absence of ciliated cells and a sparse microvillous pattern. The cells are variable in size and have a collapsed appearance. Scale bar represents 10 $\mu$ m.

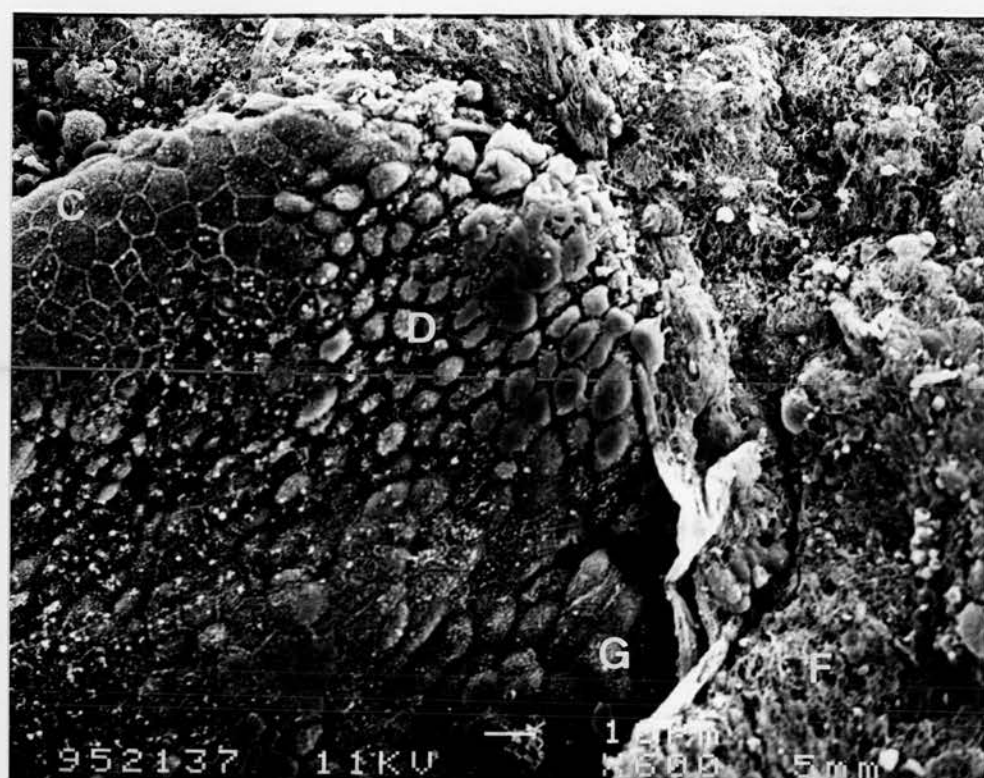


Figure 4.7.

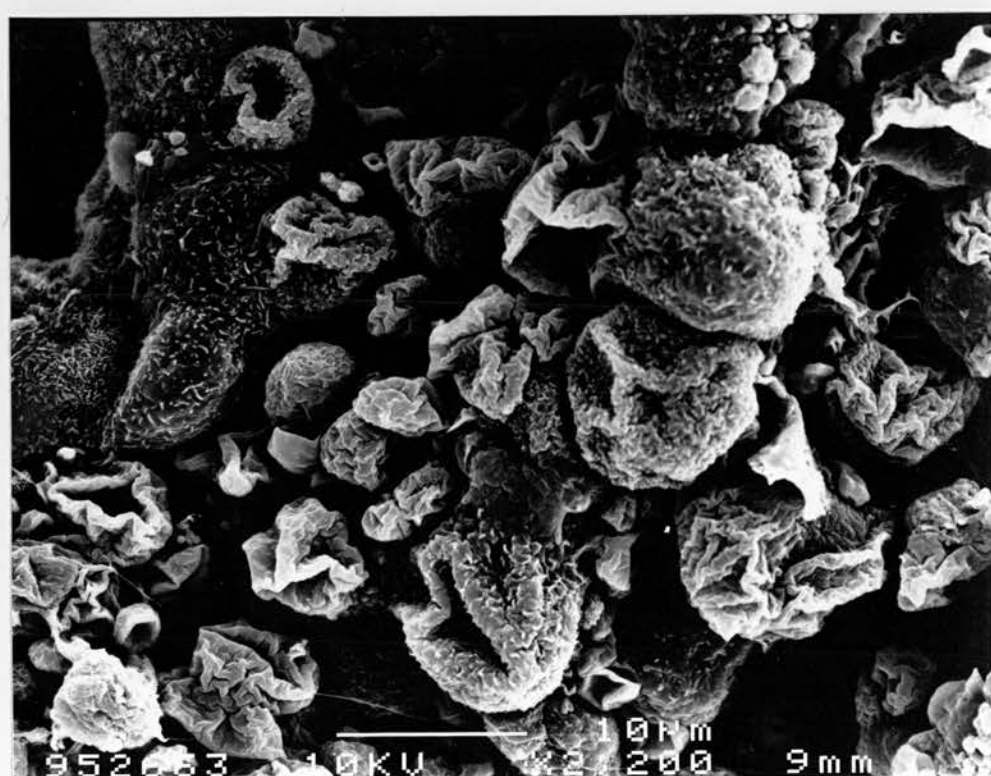


Figure 4.8.

Figure 4.9 (x330). Levonorgestrel-treated biopsy.

General view of the surface epithelium following 21 days exposure to levonorgestrel. Cilia are almost absent, microvilli are markedly reduced and the cell margins can be seen, giving a flattened cobblestone appearance to the epithelium. A gland opening (G) can be seen to the left of the picture. Scale bar represents 100µm

Figure 4.10 (x1800). Levonorgestrel-treated biopsy.

Different area of the same specimen at a higher magnification, demonstrating the variability in surface topography after treatment with levonorgestrel. The surface epithelium is devoid of surface features and demonstrates a flattened cobblestone appearance with prominent epithelial cell margins (M). A red blood cell (RBC) is seen in the centre of the field. Scale bar represents 10µm.



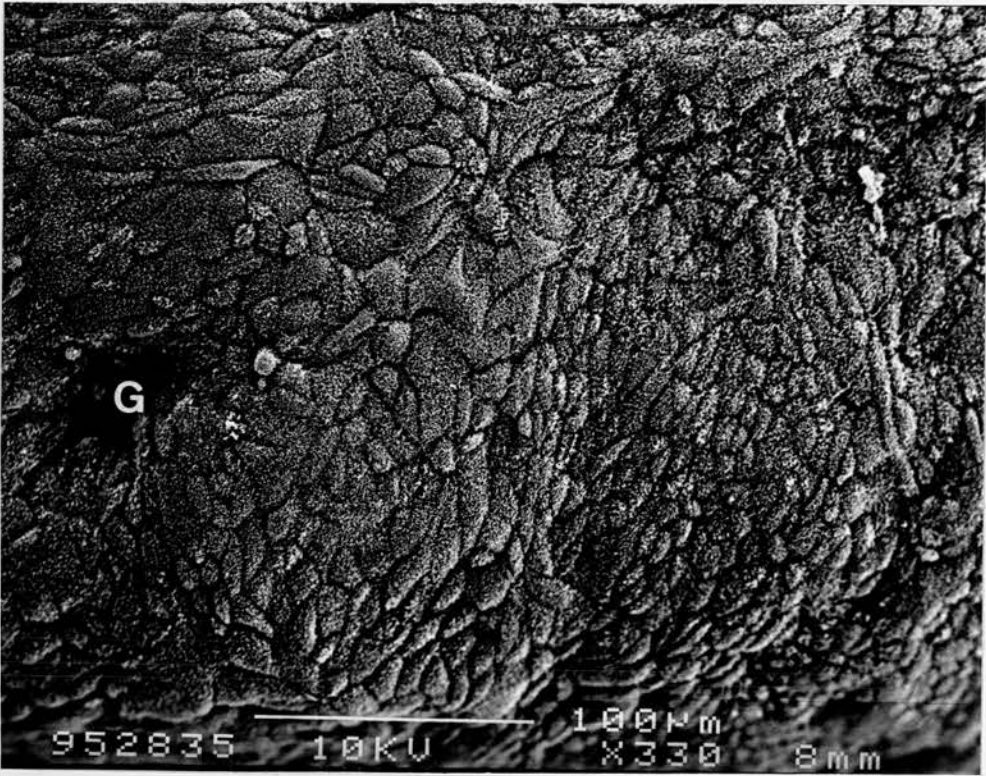


Figure 4.9.

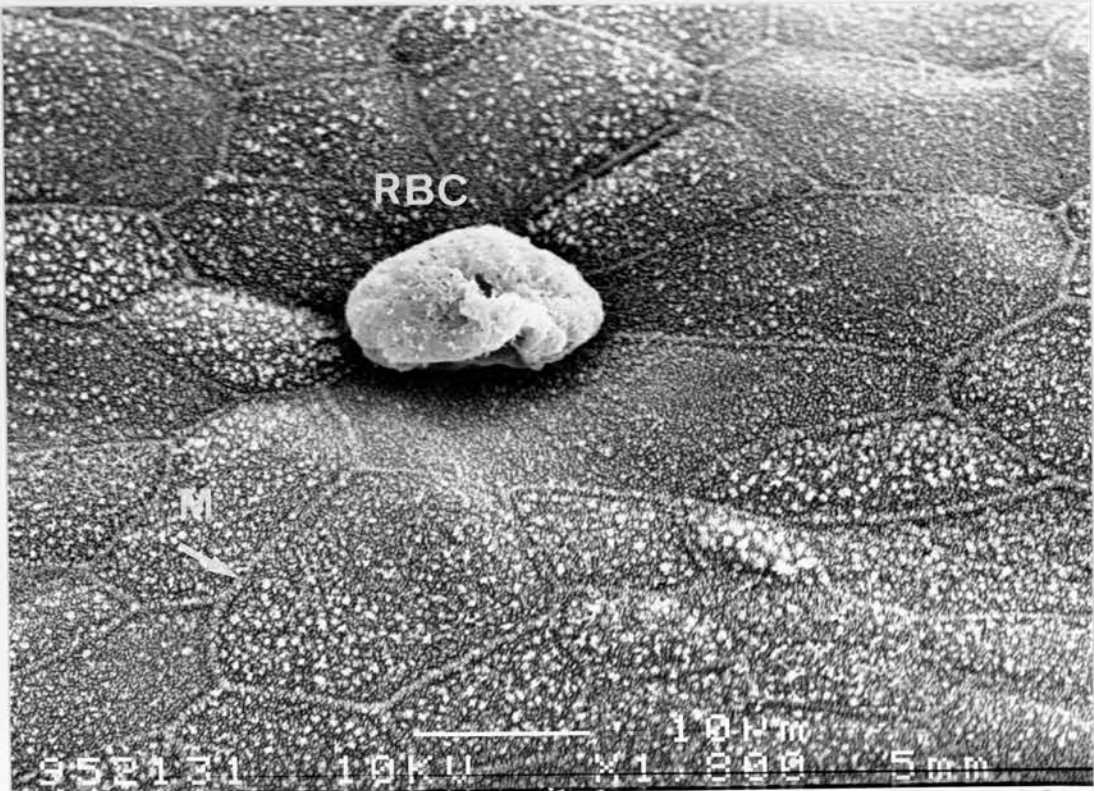


Figure 4.10.



Part Three. *Dolichos Biflorus* Agglutinin (DBA) Histochemistry of the Endometrium  
of Patients with Proven Menorrhagia, Before and After Treatment with Oral and  
Intrauterine Progestagens.

Materials and Methods

*Collection of endometrial biopsies*

Endometrial tissue was obtained from 18 healthy parous women with proven menorrhagia taking part in the clinical trial described in chapter three. The tissue was collected as described in part one of this chapter in the mid luteal phase of the cycle (median day 21, range day 15-26 days). The timing of biopsy collection is shown in table 4.4.

Eleven women received intrauterine levonorgestrel via a LNG IUS. Seven women received oral norethisterone. A second endometrial biopsy was taken in the mid-luteal phase of the first treatment cycle. The median exposure time of the tissue to intrauterine levonorgestrel was 19 days (range 15-24 days) and to oral norethisterone was 18 days (range 13-19 days). The timing of biopsy collection is shown in table 4.4.

10  $\mu$ m cryostat sections of tissue were cut, mounted onto 3-aminopropyltriethoxysilane coated slides (Sigma, UK) and air dried. Sections were fixed in acetone (BDH, UK) for ten minutes, then incubated in 0.5% hydrogen peroxide (Sigma, UK) in methanol (BDH, UK) for 30 minutes at room temperature to inactivate endogenous peroxidase and washed twice for five minutes in phosphate buffered saline (PBS) containing 10mM sodium phosphate, 120 mM sodium chloride (pH 7.5). Sections

were then incubated for 45 minutes at room temperature in a 10 µg / ml solution of DBA-biotin (Vector Laboratories, Peterborough, UK) diluted in PBS + 3% bovine serum albumin. After further rinsing in PBS for five minutes, sections were incubated for 30 minutes at room temperature with a commercially available avidin / biotin / peroxidase complex (Vectastain Elite ABC reagent: Vector Laboratories, UK), rinsed again in PBS for five minutes and DBA binding was localised using 1 mg / ml diaminobenzidine tetrahydrochloride (Sigma, UK) and 0.02% hydrogen peroxide in 50mM Tris Cl, pH7.6. Sections were washed in tap water to stop the reaction, carefully wiped dry and mounted in Immu-mount medium (Shandon, Pittsburgh, USA).

Negative control sections were treated in an identical fashion without DBA-biotin solution in PBS + 3% BSA.

Sections were examined and photographed using the appropriate condenser and a conventional bright light field microscope (Orthoplan, Leitz: Leica Ltd., UK). Staining was assessed independently by two observers and graded subjectively according to the extent of staining within individual epithelial cells and the number of cells stained within the glands or surface epithelium.

### Statistical analysis

An unpaired t-test was used to compare exposure time of the tissue to treatment in the two groups.

## Results

There was no significant difference in the exposure time of the tissue to treatment in the two groups.

In general there was an unexpectedly high level of background staining in all the specimens examined, including the controls. Preliminary studies using paraffin sections had not shown this degree of background staining.

### *Pre-treatment biopsies*

DBA was expressed in glandular and luminal epithelium, although the pattern of staining was heterogeneous, with some glands and areas of surface epithelium showing little or no staining. The pattern of subcellular localisation was also heterogeneous, with some cells showing staining confined to the apical surface, while others exhibited staining within the cytoplasm as well. There was little or no staining of secretions within the glandular lumen (figure 4.11).

### *Post-treatment biopsies*

Following treatment with either levonorgestrel or norethisterone, there was a reduction in epithelial cell height of both glands and surface epithelium, with columnar cells showing a more cuboidal pattern (see part one). The pattern of staining was similar to the pre-treatment biopsies, with heterogeneous expression of DBA between glands and between cells within the glands (figure 4.12). In general the pattern of DBA staining within glands was increased, with more cells within the gland exhibiting staining throughout the cytoplasm. As with the pre-treatment biopsies, there was little or no staining of secretions within the gland lumina. There was no obvious difference in the pattern of DBA expression between the levonorgestrel- and norethisterone-treated groups.

Figure 4.11. Photomicrograph of DBA immunostaining of a pre-treatment biopsy taken on day 21 of the cycle. Four glands (G) are seen, two with almost all glandular epithelial cells exhibiting staining throughout the cytoplasm, one with a more heterogeneous pattern of epithelial staining and one with negative staining. Scanty luminal secretions are apparent (S). Scale bar represents 25 $\mu$ m.

Figure 4.12. Photomicrograph of DBA immunostaining of a post-treatment biopsy taken following 18 days exposure to norethisterone. The tissue is less well preserved than the pre-treatment biopsy. Three glands (G) and an area of surface epithelium (S) are seen, with a flattened epithelium compared with the pre-treatment biopsy. Cytoplasmic staining is observed in all the cells within the glands and surface epithelium (S). Scale bar represents 100 $\mu$ m.

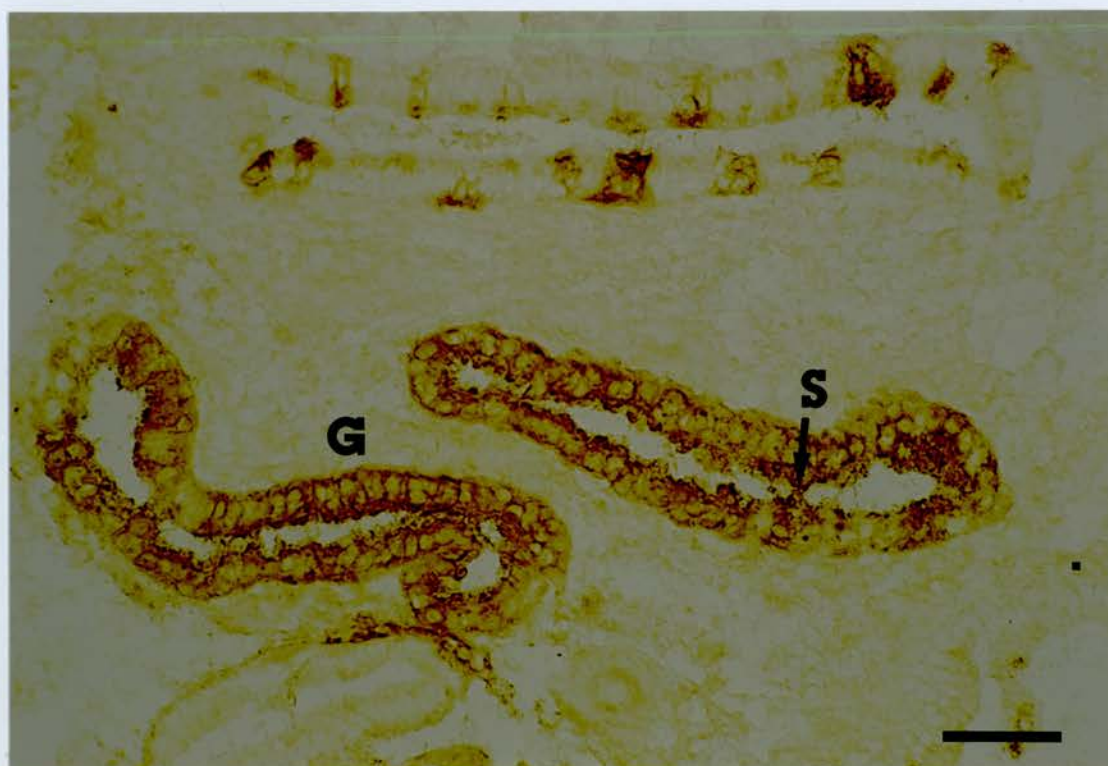


Figure 4.11

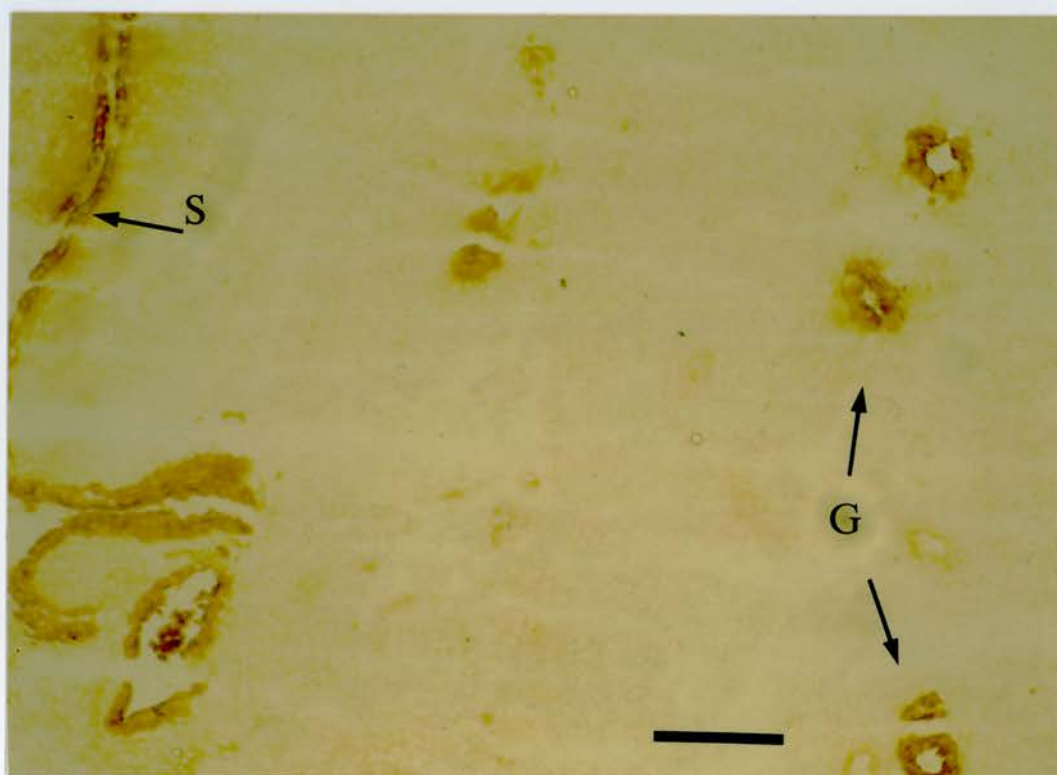


Figure 4.12.

Day of pre-treatment biopsy	Number of patients	Days exposure to progestagens	Number of patients
day 15	-	13	-
day 16	x	14	xx
day 17	-	15	x
day 18	x	16	xx
day 19	xxx	17	xx
day 20	-	18	xx
day 21	xxxx	19	xx
day 22	xxx	20	xxxx
day 23	xx	21	xx
day 24	xx	22	x
day 25	xx	23	-
day 26	-	24	-
Total	18	Total	18

Pre-treatment biopsies

Treatment biopsies

Table 4.4. Timing of endometrial biopsies in pre-treatment and treatment cycles. Pre-treatment biopsies are expressed as number of days from last menstrual period. Treatment biopsies are expressed as number of days exposure to exogenous progestagens. x=one patient.



Part Four.  $\alpha$  2 Laminin Immunocytochemistry in the Endometrium of Patients with  
Proven Menorrhagia, Before and After Treatment with Oral and Intrauterine  
Progestagens.

Materials and Methods

*Collection of endometrial biopsies*

Endometrial tissue was obtained from 19 healthy parous women with proven menorrhagia taking part in the clinical trial in chapter three. The tissue was collected as described in part one of this chapter in the mid luteal phase of the cycle (median day 21, range day 16-25). The timing of biopsy collection is shown in table 4.5.

Ten women received intrauterine levonorgestrel via a LNG IUS. Nine women received oral norethisterone. A second endometrial biopsy was taken in the mid-luteal phase of the first treatment cycle. The median exposure time of the tissue to intrauterine levonorgestrel was 19 days (range 15-22 days) and to oral norethisterone was 18 days (range 14-20 days). The timing of biopsy collection is shown in table 4.5.

10  $\mu$ m cryostat sections of tissue were cut, mounted onto 3-aminopropyltriethoxysilane coated slides (Sigma, UK) and air dried. Tissue sections were fixed in acetone for ten minutes at room temperature and washed for five minutes in phosphate buffered saline (PBS) containing 10mM sodium phosphate, 120 mM sodium chloride (pH 7.5). A commercially available kit, mouse Vectastain<sup>®</sup> Elite ABC Kit (Vector Laboratories, UK) was used for the immunohistochemical staining. Sections were first incubated in a humidified box with horse serum for 30 minutes at room temperature to remove any



non-specific binding. The blocking buffer was blotted off and the sections were incubated in a humidified box for one hour at room temperature with the primary antibody, a monoclonal antibody raised in mouse against  $\alpha 2$  laminin (Life Technologies, Paisley, UK), diluted 1:900 with the blocking serum. This concentration had been established by previous work to be the optimal dilution for this type of tissue. The sections were rinsed twice in PBS for five minutes and the secondary antibody of biotinylated anti-mouse, diluted 1:200 with blocking buffer and 1.5% human serum, was added from the Vectastain<sup>®</sup> kit (Vector Laboratories, UK). The sections were incubated in a humidified box for 30 minutes at room temperature, then rinsed twice in PBS for five minutes. Endogenous peroxidase activity was removed by incubating the sections for 30 minutes at room temperature in 0.5% hydrogen peroxide in methanol and the tissue was rinsed twice for ten minutes in PBS. The sections were incubated for 30 minutes at room temperature with the Vectastain<sup>®</sup> reagent containing a preformed avidin and biotinylated horseradish peroxidase complex. The sections were rinsed twice in PBS for five minutes and  $\alpha 2$  laminin binding was localised using 1 mg / ml diaminobenzidine tetrahydrochloride (Sigma, UK) and 0.02% hydrogen peroxide in 50mM Tris Cl, pH7.6 until a brown end product developed. Sections were rinsed in tap water to stop the reaction, counterstained with 0.2% Toluidine Blue in 30% ethanol and mounted in DPX mounting medium (BDH, UK).

Negative control sections were incubated without the primary antibody.

Sections were examined and photographed using the appropriate condenser and a conventional bright light field microscope (Orthoplan, Leitz: Leica Ltd., UK). Staining was assessed by two observers and graded subjectively according to the site and extent of staining within the specimen.

### Statistical analysis

An unpaired t-test was used to compare exposure time of the tissue to treatment in the two groups.

### Results

There was no significant difference in exposure time to treatment in the two groups. There was no  $\alpha 2$  laminin immunostaining in the negative control biopsies. Four biopsies demonstrated poor morphological preservation and were excluded from analysis. Two were pre-treatment and two post-treatment, one following norethisterone, the other levonorgestrel administration.

Endometrial blood vessels expressed  $\alpha 2$  laminin in all biopsies examined.  $\alpha 2$  laminin was expressed in the basement membrane of endometrial glands and surface epithelium in some but not all biopsies (figure 4.13). There was no difference in basement membrane  $\alpha 2$  laminin expression pre- and post-treatment. Expression of  $\alpha 2$  laminin in the stromal compartment was not uniform in the pre-treatment biopsies, with eight of the 17 biopsies examined exhibiting no stromal expression of  $\alpha 2$  laminin. In the remaining nine biopsies,  $\alpha 2$  laminin expression was mainly confined to sub-epithelial and peri-vascular areas. Following treatment,  $\alpha 2$  laminin expression was detected in the stroma of all of the biopsies examined. In 11 of the 17 post-treatment biopsies (seven levonorgestrel-treated, four norethisterone-treated), staining was no longer restricted to sub-epithelial and peri-vascular stroma, but demonstrated a more widespread distribution (figure 4.14). However even in these biopsies, the pattern of stromal staining was not uniform throughout the stromal compartment.

Figure 4.13. Photomicrograph of  $\alpha 2$  laminin immunostaining of a pre-treatment biopsy taken on day 22 of the cycle. The section is counterstained with toluidine blue. Several glands (G) are seen, with positive staining in the basement membrane. Endometrial blood vessels (BV) are also stained positive for  $\alpha 2$  laminin. Scale bar represents  $100\mu\text{m}$ .

Figure 4.14. Photomicrograph of  $\alpha 2$  laminin immunostaining of a post-treatment biopsy following 20 days exposure to intrauterine levonorgestrel. The section is counterstained with toluidine blue. Beneath the surface epithelium (SE) is an area of decidualised stroma (D), staining strongly positive with  $\alpha 2$  laminin. Scale bar represents  $50\mu\text{m}$ .



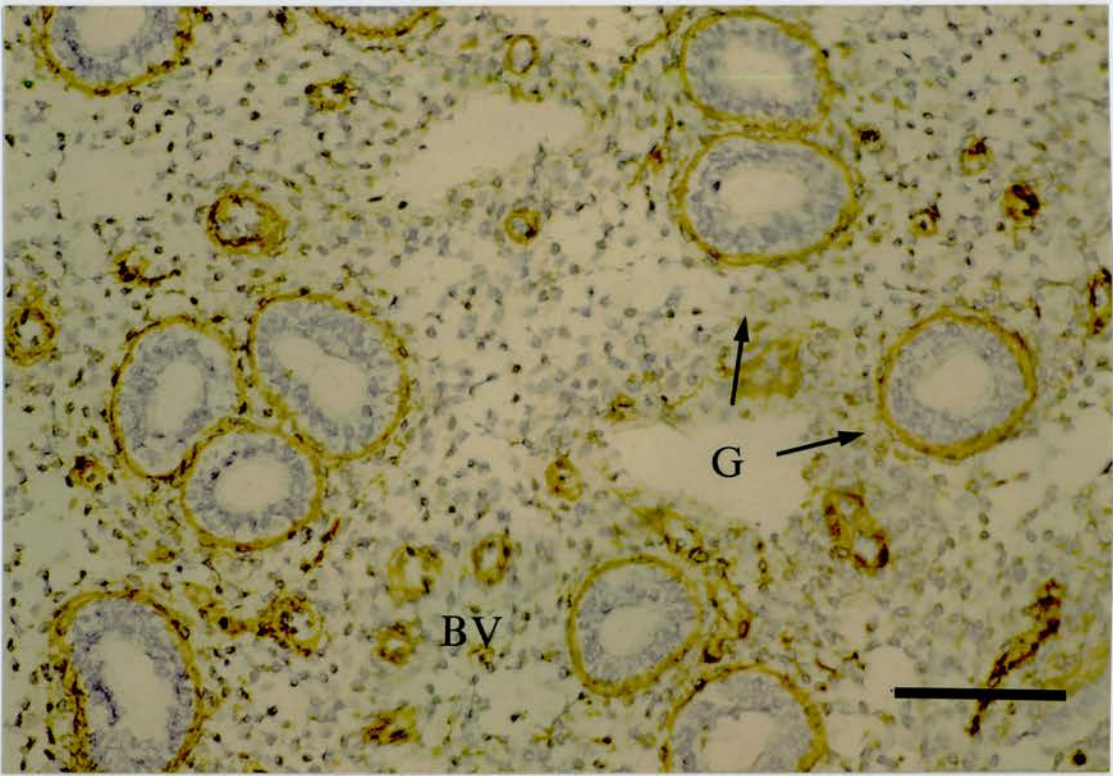


Figure 4.13.

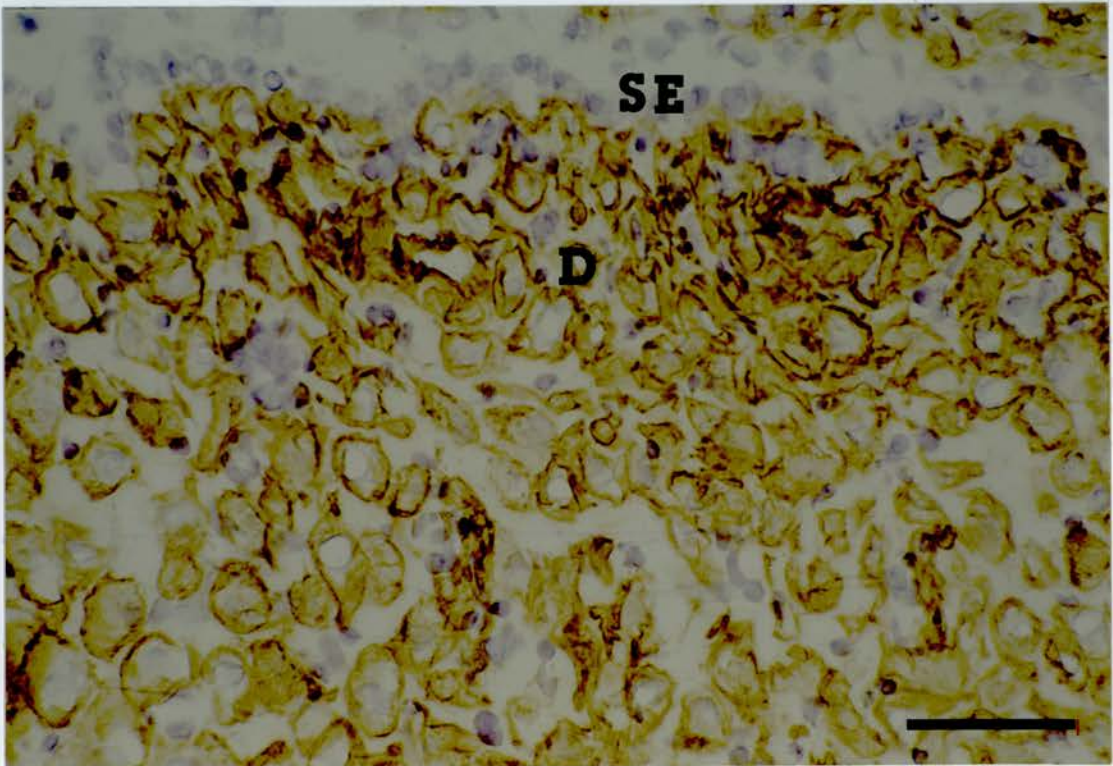


Figure 4.14.

Day of pre-treatment biopsy	Number of patients	Days exposure to progestagens	Number of patients
day 15	-	13	-
day 16	x	14	x
day 17	x	15	x
day 18	x	16	xx
day 19	xxx	17	xxx
day 20	-	18	xxx
day 21	xxxx	19	xx
day 22	xxxx	20	xxxx
day 23	x	21	xx
day 24	xx	22	x
day 25	xx	23	-
day 26	-	24	-
Total	19	Total	19

Pre-treatment biopsies

Treatment biopsies

Table 4.5. Timing of endometrial biopsies in pre-treatment and treatment cycles. Pre-treatment biopsies are expressed as number of days from last menstrual period. Treatment biopsies are expressed as number of days exposure to exogenous progestagens. x=one patient.

## Discussion

This chapter has demonstrated marked structural and functional changes occurring within the glandular and stromal compartments of the endometrium within as little as one cycle of treatment with exogenous progestagens. Glandular and surface epithelial cell height was reduced and surface morphology was altered with a reduction in the size and number of cilia and microvilli. Expression of *Dolichos biflorus* agglutinin (DBA) was maintained following treatment, suggesting that epithelial cells retain their secretory function despite the dramatic alterations to their morphology, but there was little or no evidence of intraluminal secretions. Looking at the stromal compartment, exogenous progestagens were associated with an increased expression of  $\alpha 2$  laminin.

Epithelial cell height is known to alter during the menstrual cycle (Johannisson et al, 1987; Li et al, 1988), decreasing during the luteal phase. No such correlation was apparent between epithelial cell height and day of cycle for glandular (correlation coefficient 0.16) or surface (correlation coefficient 0.10) epithelial cell height in the pre-treatment biopsies in this study. One possible explanation for this is that the biopsies were restricted to the mid luteal phase, with no reduction in epithelial cell height apparent within this small window. Another explanation may lie with the timing of the biopsies, which were timed from the first day of the last menstrual period, a method known to be more inaccurate than timing from the day of the LH surge (Li et al, 1988). The magnitude of the reduction in epithelial cell height seen following treatment with both systemic norethisterone and intrauterine levonorgestrel was greater than the variation in cell height measured prior to treatment, suggesting this was a real effect of exogenous progestagens on epithelial cells.



The scanning electron microscopy appearances of the pre-treatment biopsies were similar to those reported in another study using hysterectomy specimens (Johannisson and Nilsson, 1972), confirming the sampling technique used in this study to be a valid method of collecting biopsies for study. Following as little as three weeks exposure to exogenous progestagens, the surface morphology demonstrated defective ciliogenesis and microvilli formation, both of which have been reported following exposure to exogenous progestagens (Roberts et al, 1975; Ludwig, 1982) and in early pregnancy (Johannisson and Nilsson, 1972). Taken to its extreme, these changes could result in the flattened cobblestone epithelium noted in biopsies exposed to intrauterine levonorgestrel. This may reflect the more potent progestagen, or the direct application of the hormone to the surface endometrium, bringing about more pronounced changes than systemic administration. The collapsed appearance of the apical surface of epithelial cells has not been discussed in previous studies, although it is apparent in one study examining the effects of exogenous progestagens on baboon endometrium (Wilborn et al, 1983). The effect is unlikely to have been artefact since it was noted only in the levonorgestrel-treated biopsies, and may represent a transition between the state of defective ciliogenesis and the flattened cobblestone endometrium.

Epithelial cell secretory activity, as measured by DBA staining, was apparent in glandular and surface epithelial cells in both pre-and post-treatment biopsies. The high level of background staining noted in all the biopsies may have been a function of the cryopreservation or tissue preparation, since it was not apparent in preliminary studies using paraffin sections of endometrial tissue. Tissue preparation in the various lectin binding studies has varied and can give rise to conflicting results (Aplin, 1991). All the biopsies in this study were processed in the same way and the immunohistochemistry was performed in a single batch, so comparisons before and after treatment are valid.



In all the biopsies studied there was a heterogeneous pattern of expression, with some cells expressing DBA while neighbouring cells in the same gland did not. Heterogeneity within and between glands is a consistent feature of the endometrium revealed by lectin histochemistry (Aplin, 1991). This study examined endometrial samples taken only in the mid-luteal phase and detected little or no change in the pattern of DBA expression before and after treatment. This may suggest that DBA is already maximally expressed in the mid-luteal phase and increasing the exposure of the endometrium to progestagen does not increase expression. It is interesting to note that intracellular DBA staining accumulated in post-treatment biopsies largely in the absence of intraluminal secretions. High doses of exogenous progestagens seem thus to interrupt the mobilisation and secretion of intracellular glycoproteins from endometrial glandular cells.

Stromal expression of  $\alpha 2$  laminin was increased following exposure of the endometrium to both norethisterone and levonorgestrel. This was particularly apparent in biopsies from patients treated with intrauterine levonorgestrel, when tissue exposed to intrauterine levonorgestrel for a median of only 19 days showed a very similar pattern of  $\alpha 2$  laminin expression to that seen in first trimester pregnant decidual tissue (Gaby Kohnen, personal communication).

One feature of endometrial exposure to exogenous progestagens in this study has been the heterogeneous response of the different compartments. This was notable in the surface morphology changes, and alterations in glandular DBA and stromal  $\alpha 2$  laminin expression, and was evident whether the steroid was administered locally or systemically. This suggests that the effects of exogenous progestagens are mediated by local agents within the endometrium, which may act in a paracrine or autocrine

fashion. Two such potential agents, nitric oxide and endothelin are examined in the following chapters.

## Chapter Five

Expression of Endothelial and Inducible Nitric Oxide Synthase in the  
Endometrium of Patients with Proven Menorrhagia, Before and After  
Treatment with Exogenous Progestagens.

The role of nitric oxide (NO) in the pathophysiology of dysfunctional uterine bleeding is unknown. High dose exogenous progestagens have been demonstrated to reduce menstrual blood loss in patients with idiopathic menorrhagia (chapter three). The hypothesis constructed was that high dose exogenous steroids exert their effects on menstrual blood loss by altering the expression of endometrial nitric oxide synthase (NOS).

### Materials and methods

#### *Collection of endometrial biopsies*

Endometrial tissue was obtained from 12 healthy parous women taking part in the clinical trial described in chapter three. The tissue was collected using a Z-sampler (ZSI, Chatsworth, US) in the mid-luteal phase of the cycle (median day 21, range day 19-25), was snap-frozen in liquid nitrogen and stored at -70°C until required. The timing of biopsy collection is shown in table 5.1.

Six women received intrauterine progestagen via a levonorgestrel intrauterine system (Mirena®: Leiras Oy, Turku, Finland). Six women received oral progestagens in the form of norethisterone 5mg three times daily from day 5 to 26 of their cycle.

A second endometrial biopsy was taken in the mid-luteal phase of the first treatment cycle and was snap-frozen and stored as before. The median exposure time of the tissue to intrauterine levonorgestrel was 18 days (range 13-24 days) and to oral norethisterone was 17 days (range 13-20 days). The timing of biopsy collection is shown in table 5.1.

10µm cryostat sections of tissue were cut, mounted onto 3-aminopropyltriethoxysilane coated slides (Sigma, Poole, UK) and air-dried overnight before being stored at -70°C until further use.

#### *Immunocytochemistry for eNOS*

Tissue sections were allowed to come to room temperature, fixed in acetone for 15 minutes, air dried and rehydrated with phosphate buffered saline (PBS) containing 10mM sodium phosphate, 120mM sodium chloride (pH 7.5) for five minutes. A commercially available kit, mouse Vectastain<sup>®</sup> Elite ABC Kit (Vector Laboratories, Peterborough, UK) was used for the immunohistochemical staining. Sections were first incubated in a humidified box with 3% (w/v) immunoglobulin-free bovine serum albumin (Sigma, Poole, UK) in PBS for 20 minutes at room temperature to remove any non-specific binding. The blocking serum was blotted off and the sections were incubated in a humidified box for one hour at room temperature with the primary antibody, a monoclonal antibody raised in mouse against human endothelial NOS (Affiniti, Nottingham, UK), diluted 1:500 with the blocking serum. This concentration had been established by previous work to be the optimal dilution for this type of tissue. The sections were rinsed twice in PBS for five minutes and the secondary antibody of biotinylated anti-mouse was added from the Vectastain<sup>®</sup> kit. The tissue was incubated in a humidified box for 30 minutes at room temperature, then rinsed twice for five minutes in PBS. Endogenous peroxidase activity was removed by incubating the sections for 30 minutes in 0.3% hydrogen peroxide (Sigma, UK) in methanol. The sections were rinsed twice for ten minutes in PBS and incubated for 30 minutes at room temperature with the Vectastain<sup>®</sup> reagent containing a preformed avidin and biotinylated horseradish peroxidase complex. The sections were rinsed twice for five minutes in PBS, and eNOS binding was localised using 1mg/ml 3,3'-

diaminobenzidine tetrahydrochloride (Sigma, UK) and 0.02% hydrogen peroxide in 50mM Tris Cl, pH7.6. The sections were rinsed in tap water for five minutes to stop the reaction and counterstained with Harris haemotoxylin and eosin and mounted in DPX medium (BDH, Glasgow, UK).

Negative control slides were incubated without the primary antibody. Previous work on endometrial tissue in this laboratory had also used an irrelevant IgG<sub>1</sub> mouse monoclonal antibody against glucose oxidase from *Aspergillus niger* (Dako Ltd, High Wycombe, UK), an enzyme which is not present in mammalian tissue as a control.

#### *Immunocytochemistry for iNOS*

Immunocytochemistry for iNOS was performed as described above, using the rabbit Vectastain<sup>®</sup> Elite ABC Kit (Vector Laboratories, UK) and a polyclonal antibody to amino acids 1131-1144 of murine iNOS (Cambridge Biosciences, Cambridge, UK) as the primary antibody. Previous work had shown the optimal dilution for this type of tissue to be 1:400, diluted in blocking serum as before.

Control slides were incubated without the primary antibody.

The sections were examined and photographed using the appropriate condenser and a conventional bright light field microscope (Orthoplan Leitz: Leica Ltd., Milton Keynes, UK). Staining was assessed independently by two observers and graded subjectively according to the site and extent of staining within the specimen.

### Statistical analysis

An unpaired t-test was used to compare days of exposure to treatment between the two groups.

### Results

There was no difference in the exposure time of the tissues to treatment between the levonorgestrel and the norethisterone groups.

#### *eNOS immunocytochemistry*

There was no eNOS immunoreactivity in the control slides.

All biopsies, pre- and post-treatment, showed localisation of eNOS to endometrial blood vessels. All of the pre-treatment biopsies showed positive glandular staining for eNOS, although there was some variation in the intensity of staining between specimens (figures 5.1 and 5.2). Following treatment, glandular eNOS immunoreactivity was abolished in all of the patients treated with levonorgestrel, and in three out of six of the patients treated with norethisterone. eNOS immunoreactivity was not detected in endometrial stroma pre- or post-treatment.

#### *iNOS immunocytochemistry*

There was no iNOS immunoreactivity in the control slides.



iNOS immunoreactivity localised to glandular epithelium and some vascular smooth muscle cells. Again, there was some variation in the intensity of staining between specimens. Following treatment with norethisterone or levonorgestrel, glandular iNOS immunoreactivity was attenuated or absent in all of the specimens examined. Administration of norethisterone resulted in the appearance of small areas of decidualisation in tissues from two of the six patients, whereas a pronounced decidual reaction was seen in all the biopsies taken from the six women treated with intrauterine levonorgestrel. iNOS immunoreactivity was weakly expressed in decidualised stroma in all biopsies examined (figure 5.3 and 5.4)

The tissue sections were further compared with sections stained with  $\alpha 2$  laminin, a marker for stromal cells which have undergone decidualisation (see chapter two). The areas of iNOS expression in the post-treatment biopsies corresponded to areas of stromal decidualisation.

Figure 5.1. Photomicrograph of eNOS immunostaining in a pre-treatment endometrial biopsy taken of day 16 of the cycle. There is weak staining in the glandular epithelium (G) and in an endometrial vessel (BV). Scale bar represents 25 $\mu$ m.

Figure 5.2. Photomicrograph of eNOS immunostaining in a pre-treatment endometrial biopsy taken of day 22 of the cycle. In contrast to the previous specimen, there is no staining in glandular epithelium (G), illustrating the variation in staining between specimens. Vascular endothelial cells (BV) stain strongly positive in this specimen. Scale bar represents 50 $\mu$ m.

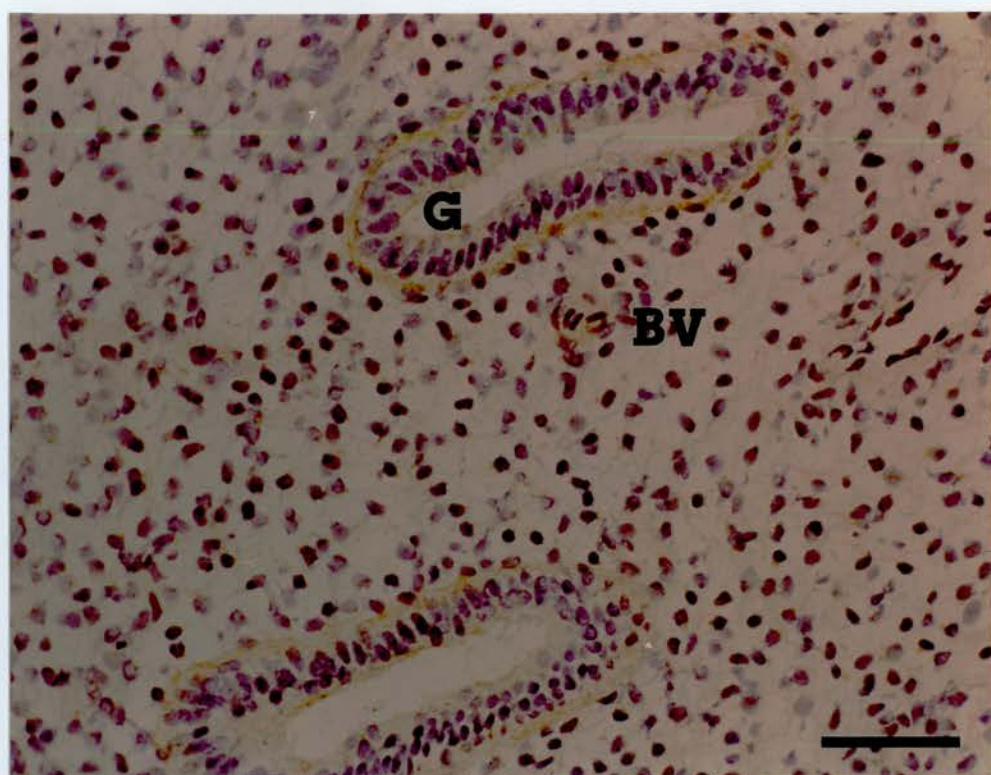


Figure 5.1.

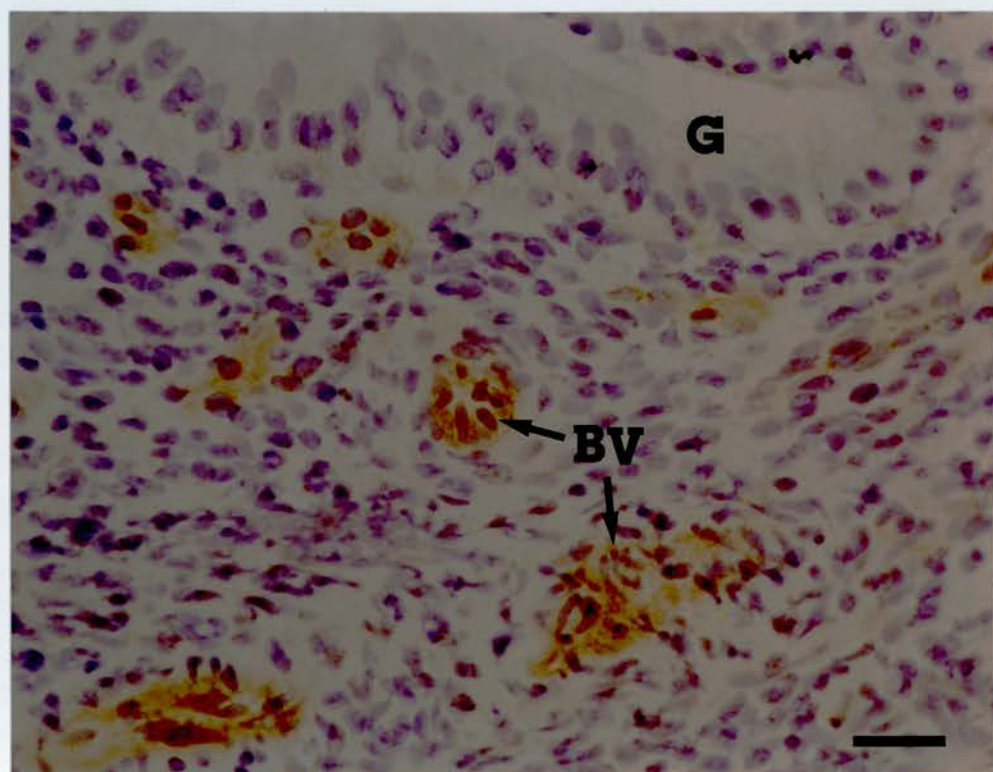


Figure 5.2.

Figure 5.3. Photomicrograph of a post-treatment biopsy following 20 days exposure to intrauterine levonorgestrel. The section is stained with haematoxylin and eosin and demonstrates the characteristic appearance of decidualised stroma immediately beneath the surface epithelium (S), with large stromal cells and a wide zone of deeply staining cytoplasm surrounding the nucleus. Scale bar represents 25 $\mu$ m.

Figure 5.4. Photomicrograph of iNOS immunostaining in parallel section to figure 5.3. There is weak staining in the decidualised stroma (D) beneath the surface epithelium (S). Scale bar represents 25 $\mu$ m.



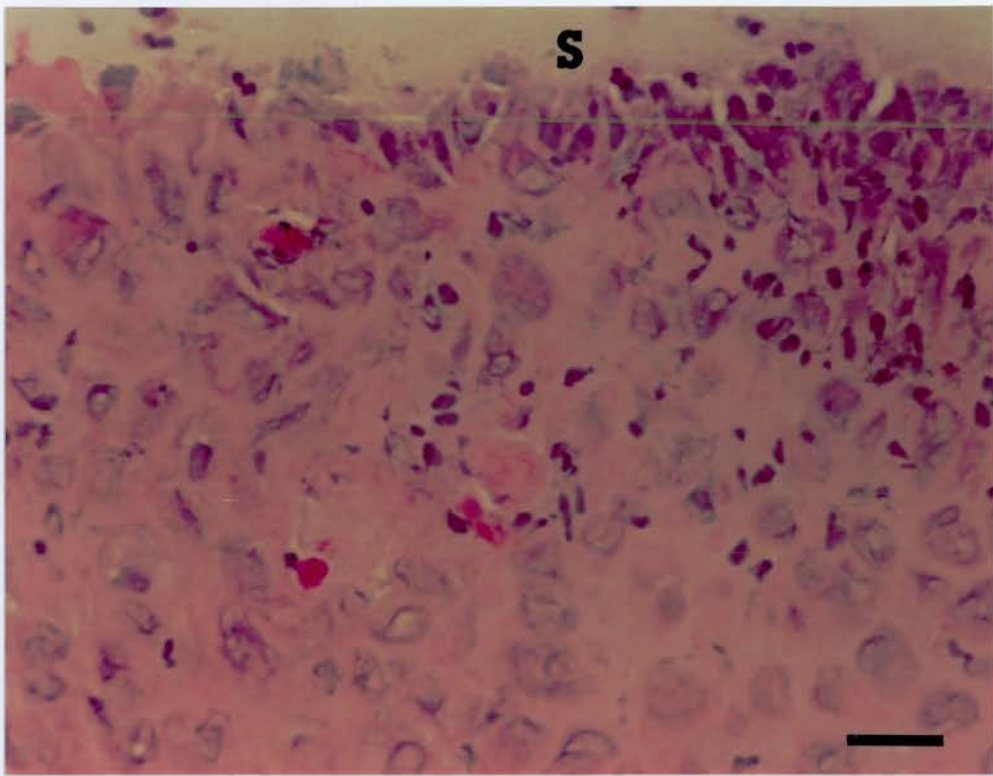


Figure 5.3.

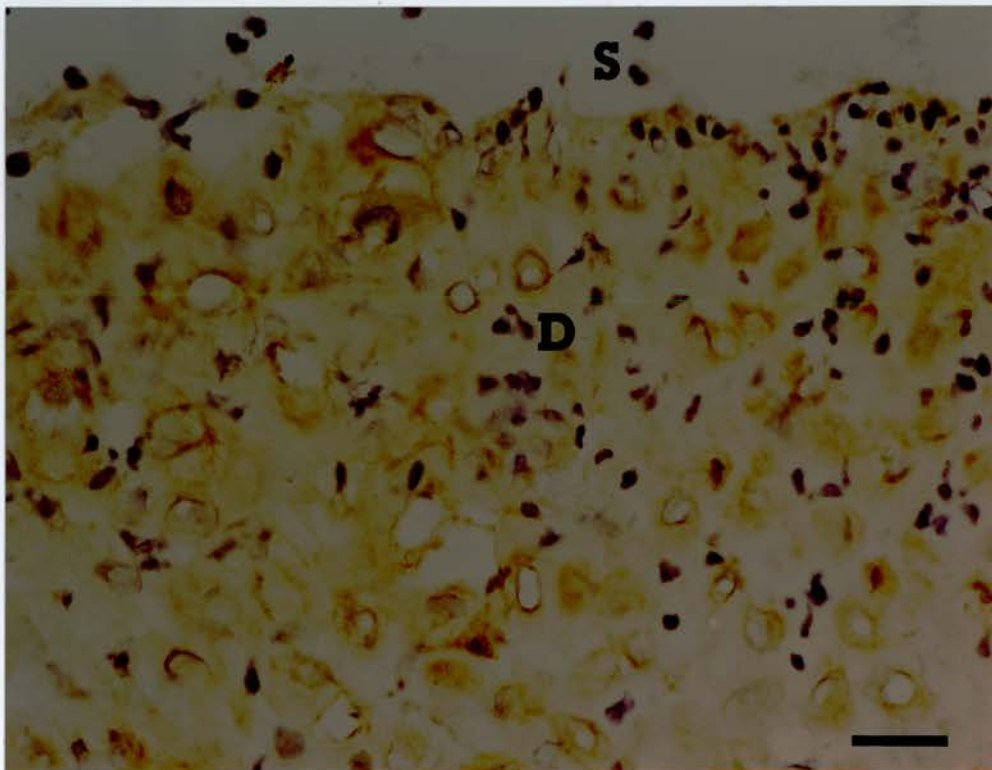


Figure 5.4.

Day of pre-treatment biopsy	Number of patients	Days exposure to progestagens	Number of patients
day 15	-	13	-
day 16	x	14	x
day 17	x	15	x
day 18	-	16	xx
day 19	x	17	xxx
day 20	-	18	x
day 21	xxx	19	-
day 22	xxx	20	xxx
day 23	-	21	-
day 24	xx	22	x
day 25	x	23	-
day 26	-	24	-
Total	12	Total	12

Pre-treatment biopsies

Treatment biopsies

Table 5.1. Timing of endometrial biopsies in pre-treatment and treatment cycles. Pre-treatment biopsies are expressed as number of days from last menstrual period. Treatment biopsies are expressed as number of days exposure to exogenous progestagens. x=one patient.

## Discussion

This study has demonstrated the differential expression of eNOS and iNOS proteins in the endometrium of patients with proven menorrhagia. eNOS protein was expressed in endometrial glandular epithelium and vascular endothelium, and iNOS protein in endometrial glandular epithelium and vascular smooth muscle cells. There was a variation in the intensity of staining for both eNOS and iNOS. Prior to treatment with both intrauterine levonorgestrel and oral norethisterone, there was no stromal expression of either eNOS or iNOS protein. Following treatment, stromal decidualisation was apparent and iNOS protein was expressed in the decidualised stroma.

There are some limitations imposed by the sampling technique. By nature of the device used to obtain the biopsies in this study, tissue specimens were a random distribution of layers of endometrium, with the deeper zona compacta being undersampled, thus there may be changes in protein expression in deeper layers compared to the more superficial ones which may not be appreciated by this sampling technique. Another potential disadvantage of this method of endometrial sampling is that the morphological structure is less well preserved. These disadvantages must be set against the major advantage of this technique which allows for repeated sampling of the same individual thus detecting any changes in protein expression following treatment.

This work confirms previous studies which have detected NOS-like immunoreactivity in human endometrium. Telfer et al detected NOS mRNA in endometrial glands, stroma and myometrial blood vessels across the cycle (Telfer et al, 1995). eNOS immunoreactivity was assessed using a monoclonal antibody raised against bovine endothelial NOS, and localised to endometrial glands and stroma in secretory phase



samples. NADPH diaphorase activity in secretory phase samples was detected predominantly in endometrial glands. This present study used a different eNOS antibody, a monoclonal antibody raised against human eNOS which detected eNOS immunoreactivity only in glandular epithelium in this type of tissue, and may thus explain the discrepancy in results. Work examining the presence of mRNA and protein for both eNOS and iNOS in 34 hysterectomy specimens using the same antibodies as in this study demonstrated no eNOS or iNOS mRNA or protein in endometrial stroma (Telfer et al, 1997) and supports the work of Tseng et al, who demonstrated intense staining in blood vessels and in endometrial glands in late secretory endometrium using NADPH diaphorase (Tseng et al, 1996). This was thought to be due to expression of eNOS, since eNOS mRNA was detected in a preparation of endometrial glands and expression was maximal in late secretory samples; eNOS mRNA was not detected in endometrial stromal preparations, in keeping with present findings. Tseng et al detected iNOS mRNA only in epithelial glands from a menstrual endometrium, with iNOS mRNA undetectable in stromal cells.

Animal studies which have demonstrated NOS activity in decidua using NADPH diaphorase activity (Moorhead et al, 1995), citrulline conversion and nitrite/nitrate production (Sladek et al, 1993). Northern blot analysis of human decidua demonstrated expression of eNOS but not iNOS mRNA (Tseng et al, 1996) and in vitro incubation of endometrial stromal cells with medroxyprogesterone acetate also failed to demonstrate expression of iNOS. This current study demonstrated expression of iNOS but not eNOS in decidualised stromal cells. One explanation for this discrepancy may be that decidual cells associated with early pregnancy express different proteins compared with the decidual cells induced by supraphysiological doses of exogenous gestagens seen in this study. Another possibility is that Northern blot analysis may not have been sensitive enough to detect decidual NOS activity. A

study on human decidua using both murine and human iNOS antibodies has confirmed the current study's results, demonstrating weak immunoreactivity on decidual cells from first trimester pregnancies (Telfer et al, 1997).

NO may play a role in the onset of menses, producing the vasodilatation which follows intense vasoconstriction of the spiral arterioles (Markee, 1940). In the study by Telfer et al in 1997, nine patients had had menstrual blood loss measured objectively prior to hysterectomy and the authors found no correlation between the intensity of eNOS immunostaining and the degree of menstrual blood loss (Telfer et al, 1997). Menstrual blood loss measurements following the first cycle of treatment of the 12 patients in this current study showed a significant reduction in all six patients receiving intrauterine levonorgestrel, and in five out of the six patients receiving oral norethisterone, correlating with reduced expression of glandular eNOS, although the number of patients was small. It is possible that the reduction in endometrial glandular eNOS expression overcame the weak expression of iNOS in decidualised stroma to produce an overall menstrual blood loss. Alternatively, changes in NOS expression and NO production may have paracrine effects on neighbouring cells, leading to an overall reduction in menstrual blood loss (Franchi et al, 1994). Six of the patients studied, one in the norethisterone group and five in the levonorgestrel group complained of frequent or continuous intermenstrual bleeding during the first cycle of treatment. These symptoms correlated with increased stromal iNOS expression, but the number of patients was small.

Although this study examined the endometrium of patients with proven menorrhagia, it should be remembered that menstruation represents a failure of fertilisation and implantation, and that the most important role of the endometrium is to facilitate the establishment of a successful pregnancy. An increase in NOS activity in decidualised stroma would be of benefit at the site of implantation in early pregnancy, catalysing the

production of NO, a potent vasodilator (Furchgott, 1988) and inhibitor of platelet aggregation and adhesion (Radomski et al, 1987 a,b) to maximise endometrial blood flow, and endometrially-derived NO may also reduce myometrial contractility (Izumi et al, 1993).

In summary, this work has demonstrated the presence of both eNOS and iNOS immunoreactivity in endometrial glands of patients with objective menorrhagia. Following treatment with exogenous progestagens, glandular eNOS and iNOS immunoreactivity was reduced or absent, but there was weak expression of iNOS in areas of decidualised stroma. The precise relationship between NO and other locally-produced mediators of endometrial function remains to be elucidated.

## **Chapter Six**

**Autoradiography for Endothelin A and B Receptors in the Endometrium of  
Patients with Proven Menorrhagia, Before and After Treatment with  
Exogenous Progestagens.**

Endothelin is a potent vasoconstrictor which acts via endothelin A and B receptors. Little is known about the role of endothelin in the pathophysiology of dysfunctional uterine bleeding (DUB). Endothelin-like immunoreactivity (ET-IR) has been demonstrated in the human uterus, at sites which may have an effect on spiral arteriole vasoconstriction (Davenport et al, 1991), and ET-IR is reduced in the endometrium of patients with menorrhagia (Marsh et al, 1996). High dose exogenous progestagens have been demonstrated to reduce menstrual blood loss in patients with DUB (chapter three). The hypothesis constructed was that high dose exogenous progestagens reduce menstrual blood loss by altering the expression of endothelin A and B receptors in the endometrium.

## Materials and Methods

### *Collection of endometrial biopsies*

Endometrial tissue was obtained from 18 healthy parous women taking part in the clinical trial described in chapter three. The tissue was collected using a Z-sampler (ZSI, Chatsworth, USA.) in the mid luteal phase of the cycle (median day 21, range day 16-25), was snap-frozen in liquid nitrogen and stored at -70°C until required. The timing of biopsy collection is shown in table 6.1.

Eight women received intrauterine progestagens via a levonorgestrel intrauterine system (Mirena®: Leiras Oy, Turku, Finland). Ten women received oral norethisterone 5mg three times daily from day 5 to 26 of their cycle.

A second endometrial biopsy was taken in the mid-luteal phase of the first treatment cycle and was snap-frozen and stored as before. The median exposure time of the

tissue to intrauterine levonorgestrel was 17 days (range 15 - 21 days) and to oral norethisterone was 15 days (range 13 - 21 days). The timing of biopsy collection is shown in table 6.1.

Binding to  $ET_A$  receptors was determined using [ $^{125}I$ ]-PD151242, an endothelin  $ET_A$  antagonist with a specific activity of 74Tbq/mM, 2000 Ci/mM at the activity reference date (Amersham International, Amersham, UK).

Binding to  $ET_B$  receptors was determined using [ $^{125}I$ ]BQ3020, an endothelin  $ET_B$  specific receptor ligand with a specific activity of approximately 74 Tbq/mM, 2000 Ci/mM at the activity reference date (Amersham International, UK).

Non-specific binding was assessed using unlabelled  $ET_A$  and  $ET_B$  ligands, gifted by Anthony Davenport, Clinical Pharmacology Unit, University of Cambridge Clinical School, Cambridge, UK.

### Endothelin receptor autoradiography

#### *Macroautoradiography*

10  $\mu$ m cryostat sections of tissue were cut, mounted onto 3-aminopropyltriethoxysilane coated slides (Sigma, Poole, UK) and air-dried overnight before being stored at  $-70^{\circ}C$  until further use. The tissue was allowed to come to room temperature and preincubated for 15 minutes in buffer containing 50mM HEPES, 5mM  $MgCl_2$  and 0.3% bovine serum albumin (pH 7.4) (Sigma, Poole, UK) to rehydrate the tissue. Sections were then incubated in HEPES buffer containing 0.1pM [ $^{125}I$ ]PD151242 or [ $^{125}I$ ]BQ3020 (Amersham, UK) for two hours at room temperature. This

concentration had been established by previous work to be the optimal dilution for this type of tissue. Non-specific binding was assessed by incubating sections in HEPES buffer containing 0.1pM [<sup>125</sup>I]-PD151242 or [<sup>125</sup>I]BQ3020 with an excess of the corresponding unlabelled ligand (1μM). Sections were washed three times for ten minutes each time in ice-cold 50mM Tris-HCL (pH 7.4), briefly rinsed in deionized water at 4°C, fixed in ice-cold formalin for ten minutes, washed again three times for five minutes each time in deionized water at 4°C and air dried.

The slides were loaded into an autoradiography cassette along with standard [<sup>125</sup>I] microscales (Amersham, UK) and exposed to Hyperfilm-βmax (Amersham, UK) for four days. The film was developed in Kodak D-19 developer (Sigma, UK) for six minutes, rinsed in tap water for 90 seconds and fixed with Kodak rapid fixer (Sigma, UK) for 12 minutes. The film was then rinsed in running tap water for 15 minutes.

The autoradiographic film was examined by two independent observers and photographed using a wide field condenser and a x1 degree objective lens in a conventional bright field microscope (Orthoplan, Leitz: Leica UK Ltd., Milton Keynes, UK).

#### *Microautoradiography*

The slides were coated in Hypercoat EM-1 emulsion (Amersham, UK) at 42°C using a wire loop to achieve as thin and uniform a coating as possible, and stored in a light-tight box containing silica gel at 4°C for six days. Slides were developed in Kodak D-19 developer for five minutes, rinsed for 30 seconds in 0.5% acetic acid, fixed for ten minutes in GBX fixer (Sigma, UK) and rinsed in running tap water for 15 minutes.



Tissue sections were counterstained with 0.2% Toluidine Blue in 30% ethanol, and mounted in DPX-mounting medium (BDH, Glasgow, UK). The sections were examined and photographed under epipolarisation with a x10 objective lens in a conventional bright light field microscope (Orthoplan Leitz: Leica Ltd., UK), and binding assessed by two independent observers as the pattern of developed silver grains in the nuclear emulsion above the tissue section.

### Statistical analysis

An unpaired t-test was used to compare days of exposure to treatment between the two treatment groups.

### Results

There was no difference in the exposure time of the tissues to treatment between the norethisterone and the levonorgestrel groups.

Interpretation of the macroautoradiographic images was limited by the sampling technique. Tissue was obtained mostly from the stratum functionalis, without the orientation seen in blocks of tissue obtained at hysterectomy. Two specimens obtained following treatment with norethisterone were fragments only and it was impossible to comment on patterns of binding in these specimens. Binding of  $ET_A$  and  $ET_B$  was apparent in all of the other macroautoradiographs, but the resolution of the macroautoradiographic images did not allow for an accurate discrimination between glands and stroma.

The specificity of binding of both [ $^{125}$ I]PD151242 and [ $^{125}$ I]BQ3020 was confirmed by the absence of binding of the labelled ligand in the presence of an excess of the unlabelled ligand.

The distribution of ET<sub>A</sub> and ET<sub>B</sub> receptors in the endometrium was assessed by microautoradiography. When the pre-treatment biopsies were examined, ET<sub>A</sub> receptors were expressed in the stroma in all specimens. There was no expression of ET<sub>A</sub> receptors in endometrial glands or surface epithelium. It was not possible to confidently localise ET<sub>A</sub> receptors to blood vessels because of the high level of stromal expression and the limited resolution of microautoradiography. ET<sub>B</sub> receptors were localised to endometrial glands in some but not all pre-treatment specimens. Surface epithelium was consistently negative. In specimens where the deeper layers had been sampled, there was positive staining for ET<sub>B</sub> in the deeper layers and negative staining in the superficial layers. There was positive stromal staining in only three of the control biopsies taken on days 21, 22 and 25 of the cycle.

Following treatment with either intrauterine levonorgestrel or oral norethisterone, there was no consistent change in the pattern of ET<sub>A</sub> receptor expression. When ET<sub>B</sub> receptors were assessed, there was expression of ET<sub>B</sub> receptors in the stroma of eight out of eight specimens treated with intrauterine levonorgestrel (figures 6.1 and 6.3) and in seven out of eight specimens treated with norethisterone. The endometrial glands showed reduced expression of ET<sub>B</sub> receptors following treatment.

The macroautoradiographic film and microautoradiographic slides were further compared with tissue sections stained with  $\alpha 2$  laminin, a marker for stromal cells which have undergone decidualisation (chapter four) (figures 6.2 and 6.4). The areas of stromal ET<sub>B</sub> expression in the post treatment biopsies corresponded to areas of stromal decidualisation.

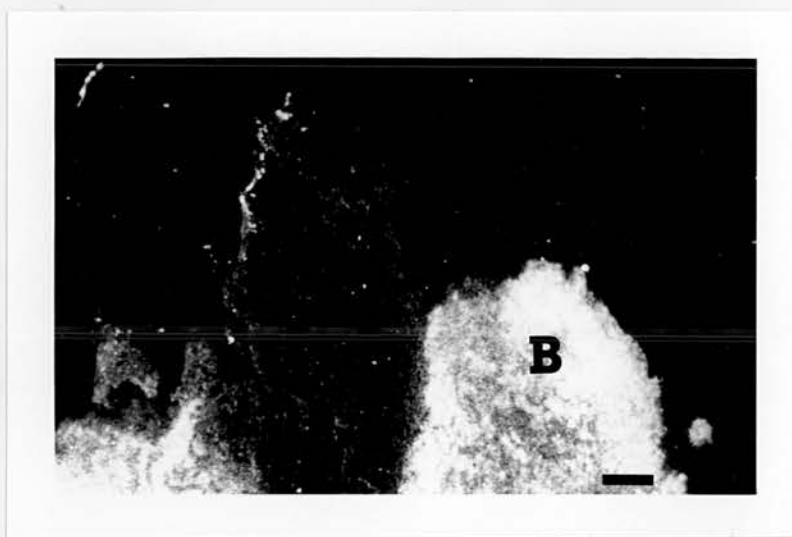


Figure 6.1. Microautoradiograph of a post-treatment biopsy following 20 days exposure to intrauterine levonorgestrel, probed with [ $^{125}$ I]BQ3020 ligand. Tangential fibre optic illumination of the section revealed areas of high intensity binding (B) which corresponded to areas of stromal decidualisation. Scale bar represents 100 $\mu$ m.

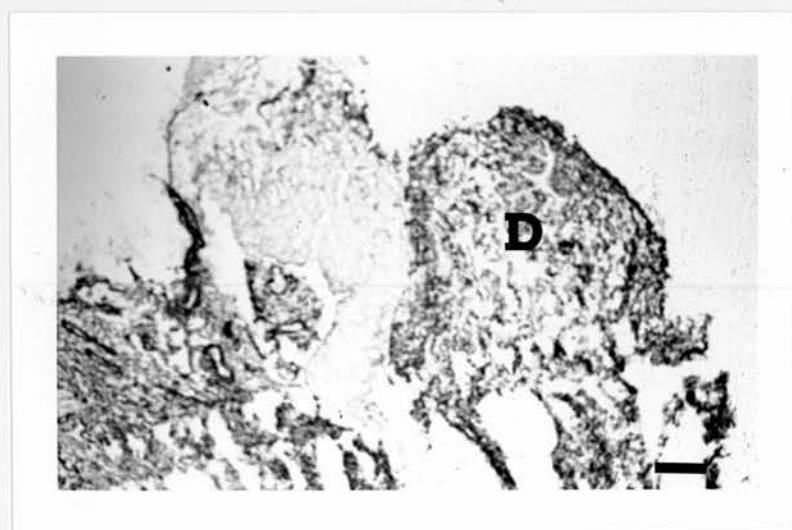


Figure 6.2. Photomicrograph of a parallel section to figure 6.1. The section is stained with  $\alpha 2$  laminin, and demonstrates areas of stromal decidualisation (D). Scale bar represents 100 $\mu$ m.

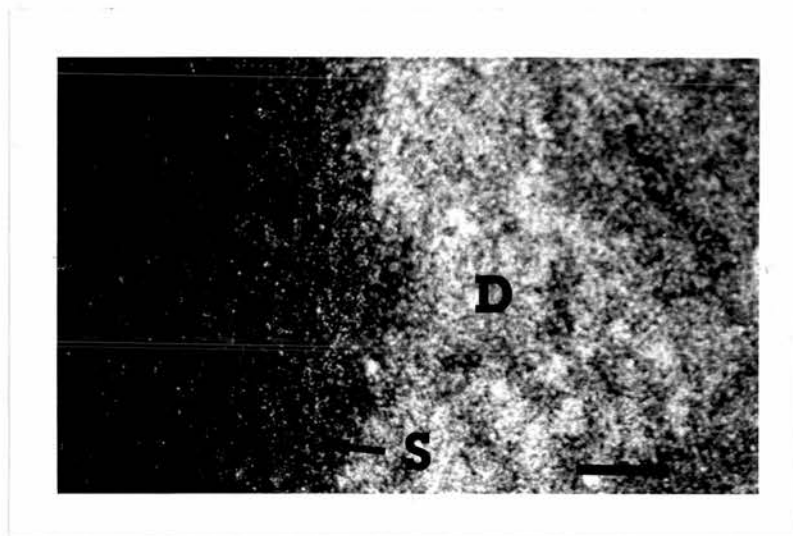


Figure 6.3. Higher magnification microautoradiograph of figure 6.1, probed with [ $^{125}$ I]BQ3020 ligand. The section demonstrates the junction between an area of decidualised (D) and non-decidualised stroma (S). Tangential fibre optic illumination revealed areas of high intensity binding which corresponded to areas of stromal decidualisation. Scale bar represents 80 $\mu$ m.

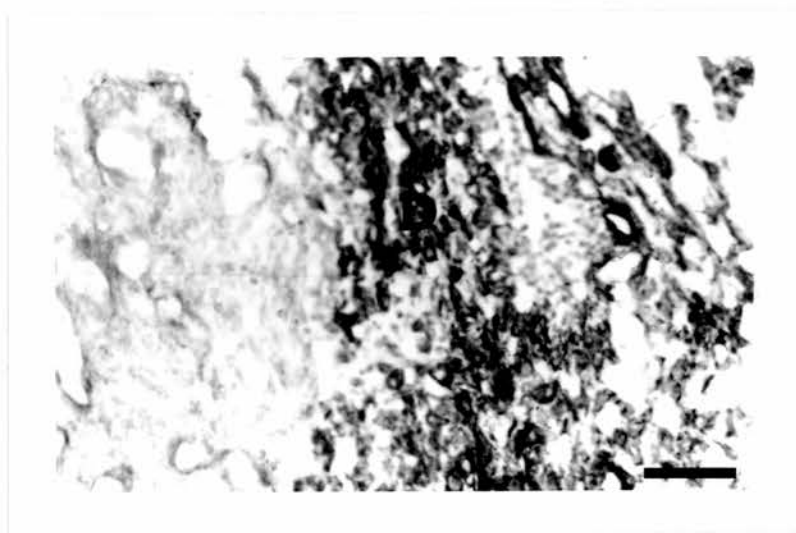


Figure 6.4. Photomicrograph of a parallel section to figure 6.3. The section is stained with  $\alpha$ 2 laminin and demonstrates stromal decidualisation (D). Scale bar represents 80 $\mu$ m.

Day of pre-treatment biopsy	Number of patients	Days exposure to progestagens	Number of patients
day 15	-	13	-
day 16	x	14	x
day 17	x	15	xx
day 18	x	16	xx
day 19	xxx	17	xx
day 20	x	18	xxxx
day 21	xxxx	19	xx
day 22	xxx	20	xxx
day 23	x	21	x
day 24	x	22	x
day 25	xx	23	-
day 26	-	24	-
Total	18	Total	18

Pre-treatment biopsies

Treatment biopsies

Table 6.1. Timing of endometrial biopsies in pre-treatment and treatment cycles. Pre-treatment biopsies are expressed as number of days from last menstrual period. Treatment biopsies are expressed as number of days exposure to exogenous progestagens. x=one patient.

## Discussion

This study demonstrates the expression of receptors for ET<sub>A</sub> and ET<sub>B</sub> in secretory phase endometrium, and following treatment with high doses of exogenous progestagens. Prior to treatment, ET<sub>A</sub> receptors localised to the stromal compartment while ET<sub>B</sub> receptors were heterogeneously expressed in the endometrial glands of secretory phase endometrium. Following treatment, ET<sub>A</sub> expression persisted in the stroma, but ET<sub>B</sub> receptor expression was apparent in areas of decidualised stroma with reduced expression in endometrial glands.

Previous studies of the expression of ET receptors across the normal cycle have shown changes in the relative levels of ET<sub>A</sub> and ET<sub>B</sub> receptor mRNA using RT-PCR and Northern blot analysis (O'Reilly et al, 1992; Kubota et al, 1995). Quantitative autoradiography confirmed these findings, and also allowed for the localisation of ET receptor subtypes, with ET<sub>A</sub> receptors localising to the endometrial stroma and ET<sub>B</sub> receptors to the endometrial glands (Collett et al, 1996). The current study examines receptor subtype localisation during the mid-luteal phase only and the findings correlate with the changing pattern of receptor subtypes across the cycle (O'Reilly et al, 1992; Kubota et al, 1995; Collett et al, 1996).

The heterogeneous pattern of glandular ET<sub>B</sub> receptor binding in this study may be explained by the sampling technique. Aspiration biopsy of the endometrium preferentially samples the superficial layers of the endometrium, with the basal layers being undersampled. In the few samples where the deeper layers were sampled, a gradient was noted with deeper layers showing an increase in glandular ET<sub>B</sub> receptor binding. This finding is in keeping with previous autoradiographic studies which have demonstrated the pattern of ET<sub>B</sub> receptor glandular binding in the mid-luteal phase to

be predominantly in the basal layers, with the superficial layers only expressing receptors from the late-luteal phase onwards (Collett et al, 1996).

High doses of exogenous progestagens induce stromal decidualisation. Work carried out on endometrium exposed to high doses of subcutaneous levonorgestrel (Norplant®) demonstrated a decrease in ET-IR in glands and luminal epithelium, and an increase in stromal ET-IR (Marsh et al, 1995) and would support the findings of the current study. In endometrium exposed to high doses of oral exogenous steroids, Northern blot analysis revealed mRNA for both ET<sub>A</sub> and ET<sub>B</sub> receptor subtypes in decidualised stroma, and decidualised stroma also expressed high levels of prepro-ET mRNA, suggesting an increase in the synthesis of receptors and endothelin (Kubota et al, 1995). The lack of an effect of treatment on the pattern of ET<sub>A</sub> receptor subtype expression in this present study may reflect the different sensitivities of autoradiography and Northern blotting techniques.

ET has been implicated as a modulator of endometrial blood flow (Davenport et al, 1991; Cameron et al, 1992), where it may play a role in the spiral arteriole vasoconstriction which precedes menstruation (Markee, 1940). Levels of prepro-ET-1 mRNA (Economos et al, 1992; Kubota et al, 1995) and big ET-IR (Ohbuchi et al, 1995) are maximal during the menstrual phase of the cycle when intense spiral arteriole vasoconstriction is required to limit menstrual blood loss (Christiaens et al, 1980) and ET has been demonstrated to be a potent vasopressor agent on uterine arteries and veins in vitro (Fried and Samuelson, 1991).

The distribution of endothelin immunoreactivity has been examined across the cycle in the endometrium of patients with menorrhagia (Marsh et al, 1996), and showed a reduction in ET-IR in glandular and luminal epithelium in patients with menorrhagia when compared to patients with a normal cycle. There was no difference in the



intensity of stromal staining. In the current study, menstrual blood loss was markedly reduced after the first cycle of treatment in 16 out of the 18 patients studied. The endometrial sampling technique used in this study did not give any information about the distribution of ET<sub>A</sub> or ET<sub>B</sub> receptors in the basal endometrium, at a site which might be expected to contribute to the action of controlling bleeding from basal arteries (Davenport et al, 1991; Cameron et al, 1992) and it may be that full-thickness specimens of endometrium would be required to investigate this effect. ET<sub>B</sub> is known to be found at high concentrations in menstrual endometrium where it is thought to play a role in the control of menstruation (Collett et al, 1996). Upregulation of ET<sub>B</sub> receptor earlier in the cycle by the administration of exogenous progestagens may have contributed to the reduced menstrual blood losses seen in this study. Nine out of the 18 patients studied complained of intermenstrual bleeding during the first cycle of treatment. There was no correlation between this symptom and a change in ET<sub>A</sub> or ET<sub>B</sub> receptor binding in the post-treatment biopsies.

The second major role for ET in decidualised endometrium may be at implantation. ET is thought to induce vasodilatation via the release of nitric oxide (NO) and prostacyclin (de Nucci et al, 1988), thus providing a mechanism for fine control of endometrial blood flow. NO can be released from vascular endothelium by a range of stimuli, including ET binding to the ET<sub>B</sub> receptor (Takayanagai et al, 1991). This current study has demonstrated an increase in the expression of ET<sub>B</sub> receptors in decidualised stroma, the same cell type which has been demonstrated to express iNOS (chapter five). ET is also known to be mitogenic for a number of tissues including rat vascular smooth muscle (Hirata et al, 1988), fibroblasts (Takuwa et al, 1989), glomerular mesangial cells (Simonsen et al, 1989) and certain types of tumour cells (Shichiri et al, 1991) and by these paracrine / autocrine actions may play a role in implantation and the establishment of early pregnancy. In vitro studies have demonstrated that ET-1

modulates the release of renin and prolactin from decidual cells (Chao et al, 1994) and that ET stimulates the production of  $\text{PGF}_{2\alpha}$  from decidual cells (Schrey et al, 1992). ET has been shown to stimulate DNA synthesis in cultured human endometrial stromal cells (Kubota et al, 1995).

In conclusion, this study has demonstrated that high doses of exogenous progestagen increase the expression of  $\text{ET}_B$  receptors in areas of stroma which have undergone decidualisation. There was no consistent change in the pattern of  $\text{ET}_A$  receptors. Endothelins may thus be involved in the regulation of endometrial blood flow at the time of implantation and menstruation, and may also play a paracrine role in the regulation of decidualised stromal cells.

## Conclusions

The complaint of excessively heavy periods is one commonly encountered in gynaecological practice and, while rarely life-threatening, is a cause of much social and psychological morbidity. The majority of patients presenting with menorrhagia have no demonstrable organic pathology and this is underlined in chapter two. Synthetic progestagens such as norethisterone are the most commonly prescribed drug for the treatment of menorrhagia in the United Kingdom (Coulter et al, 1995), yet several trials have shown them to be ineffective in their currently prescribed dosage. The effectiveness of an increased dose of progestagen is demonstrated in chapter three, with a regimen of norethisterone 5mg tds from day 5 to 26 of the cycle producing an 87% reduction in menstrual blood loss at three months. This compares to a 94% reduction in menstrual blood loss at three months achieved with a levonorgestrel intrauterine system (LNG IUS).

Exposure of the endometrium to both systemic norethisterone and intrauterine levonorgestrel for two to three weeks resulted in dramatic alterations to the epithelial and stromal compartments (chapter four). This was accompanied by reductions in MBL of 64% and 87% after one cycle of treatment for norethisterone- and levonorgestrel-treated patients respectively. Glandular and surface epithelial cell height was reduced, surface epithelial ciliogenesis was defective and the normal mobilisation and secretion of intracellular glycoproteins from endometrial glandular cells was interrupted. These findings suggest retarded development within the glandular compartment, and would be in keeping with studies which demonstrate endometrial atrophy following long term exogenous progestagen administration (Nilsson et al, 1978; Ludwig, 1982; Johannisson et al, 1982; Song et al, 1995; Pakarinen et al, 1995). In contrast, the stromal compartment showed evidence of accelerated

development, with an increase in areas of stromal decidualisation. This was particularly pronounced in endometrium exposed to intrauterine levonorgestrel. Decidualisation is a key component of menstruation, with regression of decidualised endometrium occurring prior to the onset of menses, possibly resulting in the release of vasoactive substances which start the cycle of spiral arteriole vasoconstriction-vasodilatation-vasoconstriction. Prolongation of decidualisation in areas of endometrium may thus have reduced the area of menstrual breakdown, resulting in the reduction in MBL observed following the first cycle of treatment.

Nitric oxide synthase (NOS) expression was altered in endometrium exposed to norethisterone and levonorgestrel (chapter five). These alterations were more pronounced in levonorgestrel-treated patients. Glandular eNOS and iNOS activity was attenuated or abolished, while iNOS was expressed in areas of decidualised stroma. The role of nitric oxide and NOS in the pathophysiology of dysfunctional uterine bleeding is unclear, and these observations do not readily explain the reductions in MBL. The alterations in NOS expression may be of more relevance if we consider the role of the endometrium in early pregnancy. Animal studies have demonstrated NOS activity in decidua (Moorhead et al, 1995) and decidual iNOS immunoreactivity has also been demonstrated in human decidual cells from first trimester pregnancies (Telfer et al, 1997), where it may play a role in optimising blood supply to the implanting blastocyst. By delivering high concentrations of a potent progestagen direct to the endometrium the LNG IUS appears to act as a model for the endometrial response to early pregnancy.

A role for the endothelins (ETs) in spiral arteriole vasoconstriction has been proposed (Davenport et al, 1991), and they may play a role in the pathophysiology of menorrhagia (Marsh et al, 1996). Chapter six examined the distribution of ET<sub>A</sub> and ET<sub>B</sub> binding sites in the endometrium before and after treatment with norethisterone

and levonorgestrel. No alteration in the expression of ET<sub>A</sub> was apparent following treatment, but an increase in the expression of ET<sub>B</sub> which was observed in association with decidualised stroma. ET<sub>B</sub> is known to undergo more complex changes during the menstrual cycle than ET<sub>A</sub> and is found in high concentrations at menstruation, when it is thought to play a role in the control of menstruation (Collett et al, 1996). Upregulating the expression of this receptor subtype earlier in the cycle by the administration of exogenous progestagens may thus have contributed to the reduction in MBL observed in the study patients. The appearance of ET<sub>B</sub> receptors paralleled the expression of iNOS in decidualised stroma. ET<sub>B</sub> is known to stimulate the release of nitric oxide (Takayanagai et al, 1991) and may act as a mechanism for improving endometrial blood flow at the time of implantation.

One feature of the LNG IUS is the high incidence of breakthrough bleeding (BTB) associated with the first few months of use. All 22 patients reported BTB after one cycle of treatment, with ten out of 19 still reporting the symptom at the end of three months. The increased incidence of BTB observed in LNG IUS-treated patients cannot readily be explained by these studies. There was a correlation between BTB and increased expression of iNOS, but the numbers of samples were small. Endometrial blood vessels were not examined separately in these studies and it may be that BTB is more likely to be associated with changes in the structure of blood vessels rather than alterations to their function. Alterations in microvascular density have been reported following exposure to exogenous progestagens (Rogers et al, 1993; Song et al, 1995) and long term use results in superficial venous dilatation (Ludwig et al, 1982). Of the patients who elected not to continue with the LNG IUS beyond three months, breakthrough bleeding was the commonest reason cited and this may limit the widespread acceptance of the system.

High dose exogenous progestagens offer a very effective medical approach to the treatment of menorrhagia. If oral norethisterone is to be effective, it should be prescribed at a dose of 5mg tds from day 5 to 26 of the cycle, however patient compliance is restricted by this regimen with only 22% of the patients studied electing to continue with the treatment beyond three months. The levonorgestrel intrauterine system is associated with higher rates of patient satisfaction and continuation with treatment and offers a valid alternative to surgery as long-term treatment for menorrhagia. Breakthrough bleeding is common in the first few months of use and further research is required to understand the mechanisms underlying this.

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## Appendix Two. Published Papers

The following has been published, based on the text of this thesis.

Telfer J.F, Irvine G.A, Kohnen G, Campbell S and Cameron I.T. (1997) Expression of endothelial and inducible nitric oxide synthase in non pregnant and decidualised human endometrium. *Mol Hum Reprod* 3, 69-75.

The following have been submitted for publication, based on the text of this thesis.

Irvine G.A, Campbell-Brown M.B, Lumsden M.A, Heikkilä A.M and Cameron I.T. Randomised comparative study of the levonorgestrel intrauterine system and norethisterone for the treatment of idiopathic menorrhagia.

Irvine G.A, Campbell-Brown M.B, McEwan H.P, Walker J.J and Cameron I.T. Out patient hysteroscopy and endometrial biopsy in the investigation of abnormal uterine bleeding.

# Expression of endothelial and inducible nitric oxide synthase in non-pregnant and decidualized human endometrium

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Immunocytochemistry was used to localize endothelial (eNOS) and inducible (iNOS) nitric oxide synthase in human uterine tissues collected at various stages of the menstrual cycle, after exposure to exogenous progestagens, and in early pregnancy. Endothelial NOS-like immunoreactivity was detected in all specimens in endothelial cells lining blood vessels in the myometrium and endometrium, and in endometrial glandular epithelial cells. Inducible NOS-like immunoreactivity was also demonstrated in glandular epithelial cells. For both eNOS and iNOS there was considerable variation in the intensity of epithelial cell staining between samples, which was not related to the stage of the menstrual cycle at which the tissue was collected. Messenger RNA for eNOS and iNOS was detected by reverse transcription–polymerase chain reaction (RT–PCR) using total RNA purified from isolated endometrial gland fragments. Immunoreactivity for eNOS and iNOS was not present in endometrial stroma throughout the menstrual cycle, but iNOS-like immunoreactivity was seen in decidualized stromal cells both following treatment with exogenous progestagen (intrauterine L-norgestrel) and in tissues obtained in the first trimester of pregnancy. The detection of protein and mRNA for eNOS and iNOS in normal human endometrium suggests that NO may play a role in the local control of endometrial function.

**Key words:** decidua/endometrium/nitric oxide/progestagen

## Introduction

Nitric oxide (NO) is a crucial mediator of paracrine interactions, especially within the vascular system. It is a powerful inhibitor of platelet aggregation and a potent vasodilator (Palmer *et al.*, 1987). Nitric oxide also functions as a neurotransmitter and plays a role in cell-mediated cytotoxicity (Lowenstein and Snyder, 1992). This signalling molecule is therefore likely to have an important role within the endometrium where changes in vascular function occur throughout the course of the menstrual cycle and at the time of implantation. Although it is difficult to determine which cells produce NO *in vivo*, circumstantial evidence of its production by particular cell types can be obtained by localizing the enzymes which synthesize it.

NO is produced by nitric oxide synthases (NOSs), a family of isoenzymes that catalyse the oxidation of L-arginine to nitric oxide (NO) and citrulline. There are two functional classes of NOS, based on the requirement of these enzymes for calcium (Moncada *et al.*, 1991). Inducible NOS (iNOS or type II), which is calcium-independent, binds calmodulin tightly at resting intracellular calcium concentrations. Endothelial NOS (eNOS or type III), originally described in endothelial cells, and neuronal NOS (nNOS or type I) are both dependent on calcium for activity.

The production of NO in the endometrium has not been widely addressed, but iNOS protein has been reported in endometrial epithelial cells in the mouse (Huang *et al.*, 1995) and neuronal NOS protein has been detected in rat endometrial

epithelium (Schmidt *et al.*, 1992). We have previously described the presence of eNOS protein and mRNA, and NADPH diaphorase activity, in human endometrium (Telfer *et al.*, 1995). In addition, recent work has shown that human endometrial glandular cells expressed eNOS mRNA throughout the menstrual cycle, whilst the expression of iNOS mRNA was confined to epithelial glands isolated from menstrual endometrium (Tseng *et al.*, 1996).

The onset of menstruation is characterized by intense vasoconstriction (Markee, 1940). Most of the functional endometrium is shed within 20 h of the onset of bleeding, and subsequent haemostasis is thought to be achieved not by the deposition of platelet-fibrin plugs, but by vasoconstriction (Christiaens *et al.*, 1980). Nitric oxide produced in the human endometrium may play a role in the control of menstruation (and implantation) by virtue of its roles as a vasodilator and an inhibitor of platelet aggregation. Animal studies have suggested that NO, acting via second messenger cGMP, may contribute to the maintenance of uterine quiescence during pregnancy (Natuzzi *et al.*, 1993; Sladek *et al.*, 1993). Thus NO produced in the endometrium may also have an effect on the underlying myometrium.

The aim of the present study was to determine the localization of eNOS and iNOS protein in human endometrium by immunocytochemistry, and to detect the presence of mRNA for eNOS and iNOS using the reverse transcription–polymerase chain reaction (RT–PCR) on enriched preparations of glandular epithelium. The effect of ovarian steroids on the expression

of NOS in the endometrium was investigated by immunocytochemical analysis of tissue collected both at different time points in the normal menstrual cycle and from women receiving exogenous progestagens (oral norethisterone or intrauterine L-norgestrel) for the treatment of dysfunctional uterine bleeding. Localization of eNOS and iNOS in decidualized stroma was also studied in tissues from women with normal pregnancies undergoing induced abortion in the first trimester.

## Materials and methods

### *Collection of tissue from women with normal menstrual cycles*

Tissue was obtained from 34 pre-menopausal women with regular menstrual cycles undergoing hysterectomy for benign disease at the West Glasgow Hospitals University NHS Trust and Glasgow Royal Infirmary University NHS Trust, UK. In nine cases menstrual blood loss was measured by the alkaline haematin method (Hallberg and Nilsson, 1964). Informed consent was obtained in each case and the study was approved by the local ethics committees.

Histological assessment of endometrial morphology was carried out by local pathologists according to standard criteria (Noyes *et al.*, 1950).

### *Collection of tissue from menorrhagic women treated with exogenous progestagens*

Endometrial biopsies were collected from 12 women with regular menstrual cycles and objectively measured menorrhagia (menstrual blood loss >80 ml per month, median 99 ml, range 83–245 ml). Six women subsequently received oral norethisterone (5 mg three times daily from days 5 to 26 of the cycle) and six received L-norgestrel, as the L-norgestrel intrauterine system (LNG-IUS, Mirena®; Leiras Oy, Turku, Finland). The LNG-IUS, which releases 20 µg LNG daily, was inserted within 5 days of the onset of menstruation. Endometrial biopsies were taken using a Z-Sampler endometrial suction curette (Zinnanti, Chatsworth, CA, USA) at a median of 21 days (range 19–25) after the onset of menstruation during a pre-treatment control cycle. A second post-treatment biopsy was taken after a median of 18 days (range 13–24) exposure to L-norgestrel and 17 days (range 13–20) exposure to norethisterone.

### *Collection of first trimester decidua*

Decidual tissue was collected from 10 women with normal pregnancies undergoing surgical termination in the first trimester.

### *Isolation of endometrial gland fragments*

Biopsies of endometrium were transported to the laboratory in cold Roswell Park Memorial Institute (RPMI) 1640 medium containing 100 IU/ml penicillin and 100 µg/ml streptomycin (Life Technologies, Paisley, UK). The tissue was washed three times in RPMI medium and chopped into small pieces using a sterile scalpel. Tissue pieces were placed in a sterile tube with 1 mg/ml collagenase (Type I; Sigma, Poole, Dorset, UK) in RPMI medium, supplemented with penicillin and streptomycin, and incubated at 37°C for 2–4 h. Undigested tissue was removed using a sterile 400 µm woven polyester mesh (Lockertex; Locker Wire Weavers Ltd., Warrington, UK) and the filtrate was collected. This filtrate was passed through a 30 µm mesh and trapped epithelial gland fragments were back-washed using sterile Dulbecco's phosphate-buffered saline (D-PBS, Life Technologies).

### *Immunocytochemistry for eNOS*

Tissue biopsies obtained at hysterectomy, comprising endometrium and myometrium, were fixed in 10% neutral buffered formalin (BDH, Poole, Dorset, UK) and embedded in paraffin. Sections were cut (5 µm thick) and mounted on silane-coated slides, heated to 60°C for 35 min, deparaffinized in xylene and rehydrated in a graded alcohol series. The sections were pre-incubated with 3% (w/v) immunoglobulin-free bovine serum albumin (BSA; Sigma) in PBS (10 mM sodium phosphate, pH 7.5, 120 mM sodium chloride) for 20 min at room temperature. They were then incubated for 1 h at room temperature with a monoclonal antibody against human eNOS (Affiniti, Nottingham, UK), diluted 1/500 in 3% BSA. Sections were then washed in 0.1% Triton-X100, followed by two washes in PBS. Antibody binding was detected with an anti-mouse immunoglobulin peroxidase kit (Vectastain Elite ABC Kit; Vector, Peterborough, UK) in which the biotinylated bridging antibody was diluted in 3% (w/v) BSA and 1.5% (v/v) normal human serum (Sigma). After application of the biotinylated antibody the sections were washed in PBS and placed in 0.3% H<sub>2</sub>O<sub>2</sub> (Sigma) in methanol for 30 min at room temperature. The sections were then washed thoroughly in PBS and incubated for 30 min with avidin DH/biotinylated horseradish peroxidase H reagent (Vector) in PBS. Immunoreactive eNOS was localized using 1 mg/ml diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0.02% H<sub>2</sub>O<sub>2</sub> in 50 mM Tris-HCl, pH 7.6. Sections were washed in distilled water, counterstained with Harris haematoxylin and mounted in DPX (BDH).

Negative control sections were incubated without primary antibody, or with an irrelevant IgG<sub>1</sub> mouse monoclonal antibody against glucose oxidase from *Aspergillus niger* (Dako Ltd., High Wycombe, UK), an enzyme which is not present in mammalian tissue.

### *Immunocytochemistry for iNOS*

Immunocytochemistry was performed on paraffin-embedded sections using a polyclonal antibody raised against amino acids 1131–1144 of murine iNOS (Cambridge Biosciences, Cambridge, UK) diluted 1/400 in PBS. Antibody binding was detected using an anti-rabbit immunoglobulin (Ig)G peroxidase kit (Vectastain Elite ABC Kit; Vector) and DAB substrate (Sigma), according to the manufacturer's instructions. Control slides were incubated without primary antibody. Human DLD-1 colorectal adenocarcinoma cells (European Collection of Animal Cell Cultures, Porton Down, UK), which have previously been shown to express iNOS (Sherman *et al.*, 1993), were cultured in RPMI 1640 (Life Technologies) supplemented with 10% heat inactivated fetal calf serum, 100 µg/ml interferon (Sigma), 10 ng/ml tumour necrosis factor (Sigma) and 0.5 ng/ml interleukin-1 (Sigma) for 24 h, and used as a positive control.

Immunocytochemistry was also performed on frozen sections with a polyclonal anti-human iNOS antibody raised against amino acids 1135–1153 (Santa Cruz, Heidelberg, Germany). The antibody was diluted 1/200 in PBS and immunocytochemistry was carried out using an anti-rabbit IgG peroxidase kit (Vectastain Elite ABC Kit; Vector) according to the manufacturer's instructions.

### *Detection of endothelial cells using Ulex europaeus agglutinin 1 (UEA 1) lectin*

Lectin binding was carried out on 10 µm frozen sections of endometrium and myometrium mounted on silane-coated slides. The sections were fixed in acetone at room temperature for 20 min, air-dried and rehydrated in 10 mM HEPES, pH 7.5, 0.15 M NaCl, then placed in 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 min to remove endogenous peroxidase activity. Biotinylated *Ulex europaeus* agglutinin 1 (UEA 1) lectin (5 µg/ml, Vector) was applied to the section in HEPES, NaCl buffer for 1 h at room temperature. Avidin-biotin-peroxidase complex



(Vectastain Elite ABC Kit; Vector) and DAB substrate were then used to detect lectin binding.

#### Identification of decidual stromal cells in first trimester decidua

Cryostat sections were fixed in acetone at 4°C for 10 min, air-dried and rinsed in PBS for 10 min. Decidual cells were identified with a polyclonal antibody against vimentin (Euro-Path Ltd, Bude, UK). Glands and trophoblast were localized using a monoclonal antibody against cytokeratins (clone MNF 116, Dako, High Wycombe, UK). The sections were pre-incubated for 20 min in D-PBS supplemented with 1.5% BSA. The primary antibodies were applied for 60 min (anti-cytokeratin 1:400; anti-vimentin 1:130, diluted in D-PBS supplemented with 1.5% BSA). After washing in D-PBS 3 times for 5 min, biotinylated secondary rabbit anti-mouse antibody (1:500, Dako UK) or biotinylated goat-anti rabbit antibody (Vector) were applied for 30 min. Sections were then incubated in 1% H<sub>2</sub>O<sub>2</sub> in absolute methanol for 10 min to inactivate endogenous peroxidase followed by incubation with streptavidin-peroxidase-conjugate (StreptAB-complex/HRP; Dako) for 20 min. The streptavidin-biotin complex was visualized with DAB (Sigma). Negative control sections were treated in the same way as for eNOS.

#### Reverse transcription-polymerase chain reaction

Endometrial gland fragments and DLD-1 cells were centrifuged at 100 g for 5 min and lysed in Trizol reagent (Life Technologies). Total RNA was prepared by solvent extraction following the manufacturer's instructions.

Reverse transcription (RT) was carried out on 2 µg of total RNA extracted from gland fragments of three proliferative and three secretory endometria and DLD-1 cells. The RNA was annealed to 250 ng of pd(T) 19–24 (Pharmacia, Milton Keynes, UK) by heating to 70°C for 10 min. After chilling on ice, 1 IU RNAGuard (Pharmacia, Milton Keynes), 1 mM dNTPs, first strand buffer (Life Technologies) and 200 IU of SuperScript II RNase H<sup>-</sup> reverse transcriptase (Life Technologies) were added, made up to a total volume of 20 µl with water and incubated at 37°C for 1 h. The reaction was terminated by heating to 80°C for 10 min. cDNA products were stored at –20°C until amplification.

Polymerase chain reaction (PCR) was undertaken with primers (Oswell DNA Service, Southampton, UK) for human eNOS which spanned exons 8–11 (Marsden *et al.*, 1993); 5'-CAG TGT CCA ACA TGC TGC TGG AAA TTG-3' (1004–1030) and 5'-TAA AGG TCT TCT TCC TGG TGA TGC C-3' (1490–1464, Weiner *et al.*, 1994). Amplification of iNOS was carried out with primers (P.Romanowski, L. Wallman, and D.J. Williamson, St Vincent's Hospital, Darlinghurst, Australia; personal communication) which spanned exon-intron boundaries (Chartrain *et al.*, 1994); 5'-GGA ATT CAC TCA GCT GTG CAT CG-3' (1077–1099) and 5'-GTT TCC AGG CCC ATT CTC CTG C-3' (1433–1412). Amplifications were carried out using a programmable thermal cycler (TLA-2 Hybaid, Teddington, UK) in a total volume of 50 µl containing 60 mM Tris-HCl, pH 8.5, 15 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 250 M dNTPs, 0.5 M of each primer and 5 IU of *Taq* polymerase. A HotWax Mg<sup>2+</sup> bead (Invitrogen, Abingdon, UK) was added to each tube. Amplifications were carried out with an initial denaturation step at 94°C for 2 min, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. The final cycle was followed by strand extension at 72°C for 10 min. Amplified products were analysed by electrophoresis using 1.5% agarose gels and visualized by ethidium bromide staining.

The PCR products were sequenced with an ABI PRISM dye terminator cycle sequencing kit (Perkin Elmer, Warrington) using AmpliTaq DNA polymerase, FS and the sequencing products were

analysed on an automated 373A DNA sequencer (Applied Biosystems, Warrington, UK)

## Results

### eNOS and iNOS immunocytochemistry across the normal menstrual cycle

Immunocytochemistry for eNOS and iNOS was carried out on paraffin sections from 21 women. Six were in the proliferative phase of the cycle, 11 were in the secretory phase [early secretory (days 14–18) *n* = 6, mid-secretory (days 19–24) *n* = 3, late secretory (days 25–28) *n* = 2] and 4 were in the menstrual phase.

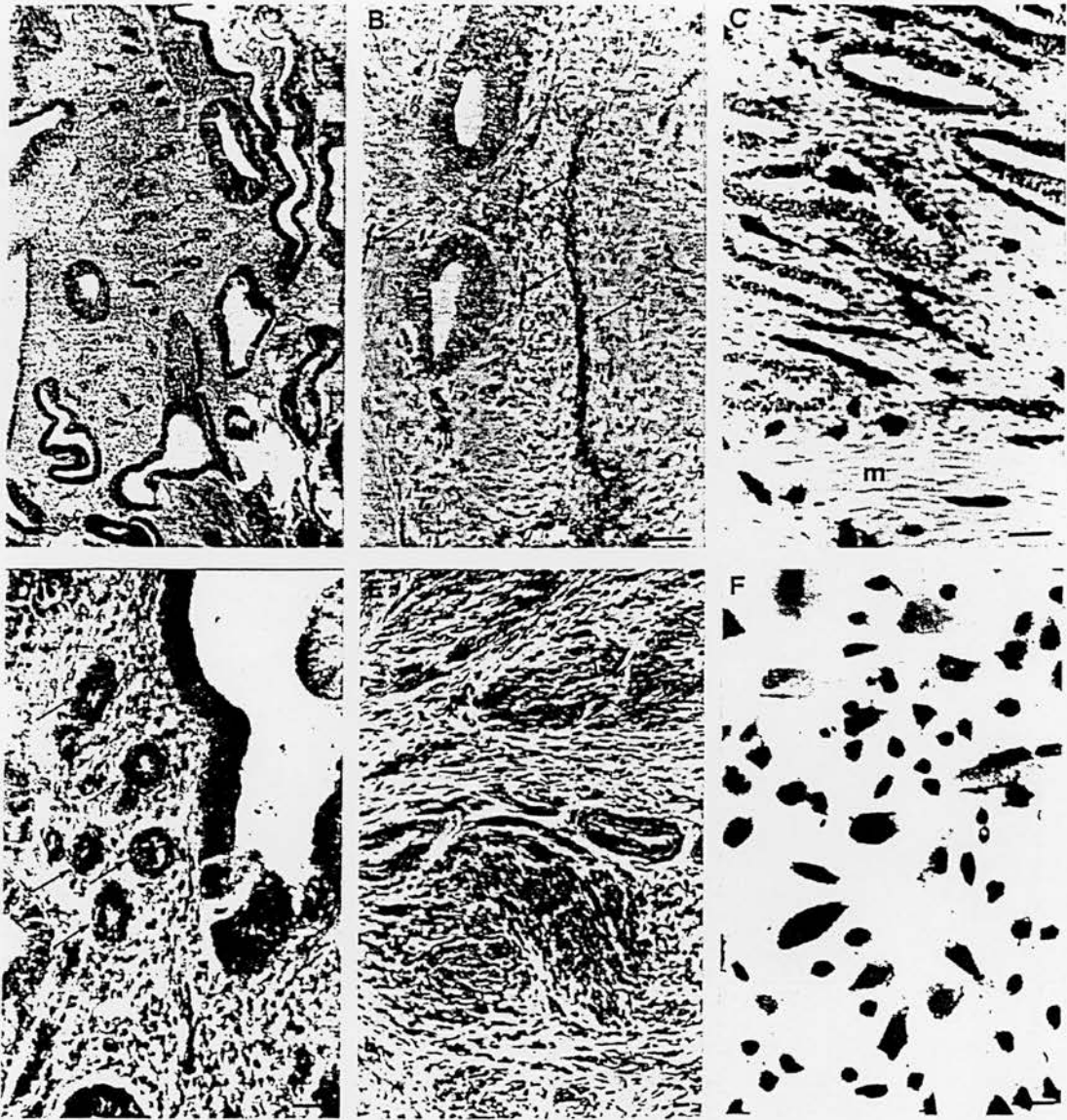
Endothelial NOS protein was detected in endothelial cells lining blood vessels (Figures 1A,B) in the myometrium and endometrium, and in endometrial glandular epithelial cells in all specimens examined. UEA 1 lectin staining confirmed that eNOS antibody stained endothelial cells (Figure 1C), but suggested that eNOS protein was not present in all endothelial cells. There was a difference both in the intensity of staining of endothelial cells and the number of blood vessels which stained positive for eNOS in endometrium and myometrium between different sections. There was also inter-individual variation in the intensity of eNOS antibody staining in glandular epithelial cells which was not related to the stage in the menstrual cycle.

In five of the 21 specimens there was marked variation in the intensity of staining of endometrial epithelial cells in the same specimen, ranging from very faint in the functional endometrium to intense in the basal region. This gradation in staining was observed in four secretory phase samples and in one sample taken in the late proliferative phase. Five tissue samples which showed no such gradation exhibited a marked reduction in staining of their surface epithelium.

Immunolocalization of eNOS on cryosections from 11 specimens confirmed eNOS was present in vascular endothelium and glandular epithelium. Although the pattern of localization was similar to that obtained on wax sections, the staining on cryosections was less extensive within the cytoplasm of individual epithelial cells.

Amongst the nine women in whom menstrual blood loss was measured before hysterectomy, five had a normal monthly blood loss (median 35 ml, range 28–76 ml) and four had menorrhagia (median 202 ml, range 98–265 ml). The intensity of immunostaining for eNOS in these samples did not appear to be related to the degree of menstrual blood loss.

Inducible NOS-like immunoreactivity was investigated in paraffin sections using the murine polyclonal antibody. Immunostaining was not present in endothelial cells, but was detected in glandular epithelial cells (Figure 1D). Once more there was a considerable difference in the intensity of staining between samples which was not related to the stage of the menstrual cycle. As with the eNOS immunocytochemistry, a gradation in staining was also observed between basal and functional layers of the endometrium in four biopsies, two of which were obtained in the proliferative phase and two in the early to mid-secretory phase of the menstrual cycle. Immunostaining of vascular smooth muscle in the myometrium,



**Figure 1.** Localization of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) protein in human uterus. (A) eNOS localized to the endothelium of a spiral arteriole (arrows) in a secretory phase specimen and also to the glandular epithelium with variable intensity. (B) eNOS was found in glandular epithelium and endothelial cells of microvessels (arrows) in menstrual tissue. (C) Localization of UEA 1 binding glycans in endometrium and myometrium (m) confirmed the vascular localization of eNOS. (D) iNOS was localized in glandular epithelium and the smooth muscle of spiral arterioles (arrows), but was absent from the microvascular endothelium. (E) iNOS was also expressed in myometrial smooth muscle and vascular smooth muscle within the endometrium. (F) Human DLD-1 adenocarcinoma cells stimulated with cytokines expressed iNOS and acted as a positive control for the anti-murine iNOS antibody. Nuclei were visualized with haematoxylin. The negative controls, without primary antibody or with an irrelevant primary antibody (see Materials and methods) exhibited no reactivity. Bar = 30 µm.

and of the myometrium itself, was seen in tissues from nine women (Figure 1E). Control cultures of human DLD-1 cells treated with cytokines stained intensely with the anti-murine iNOS antibody (Figure 1F).

The pattern of immunolocalization obtained with the anti-human iNOS antibody on cryosections was identical to that obtained with the anti-murine iNOS antibody on paraffin sections.

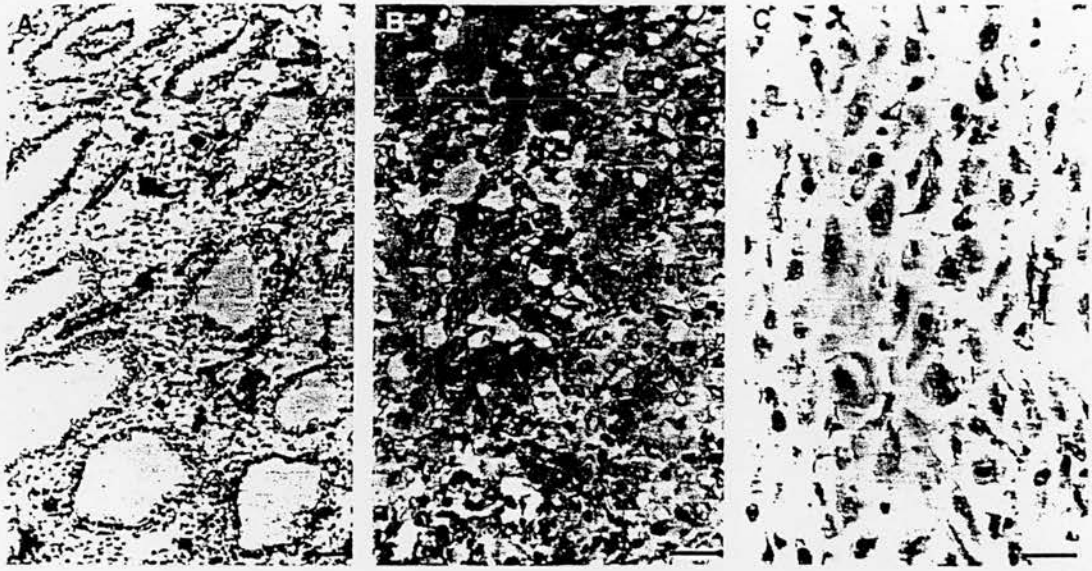
**Immunocytochemistry for eNOS and iNOS after treatment with exogenous progestagens**

The pattern of localization of eNOS and iNOS protein in the pre-treatment biopsies of 12 menorrhagic women was not

distinguishable from that observed in the biopsies from the 21 who underwent hysterectomy for benign disease. Endothelial NOS-like immunoreactivity was confined to vascular endothelium and glandular epithelium, whilst iNOS-like immunoreactivity was seen in glandular epithelium and in some vascular smooth muscle cells. Though immunoreactivity was present in all sections, there was marked variability in the intensity of staining for both vascular and epithelial cells. NOS-like immunoreactivity was not detected in endometrial stroma.

Administration of norethisterone resulted in the appearance of small areas of decidualization in tissues from two of the six patients, whereas a pronounced decidual reaction was seen in all endometrial specimens taken from the six women





**Figure 2.** Expression of nitric oxide synthase (NOS) in endometrium after exposure to exogenous progestagens. (A) Endothelial NOS localized to blood vessels after exposure to oral norethisterone for 21 days. The glandular epithelium was reduced in height and in this biopsy did not express eNOS. (B) A highly decidualized patch of endometrial stroma after exposure to LNG-IUS showing weak expression of inducible NOS (iNOS) within the stromal cells. (C) Stromal cells within first trimester decidua also expressed iNOS. The negative controls, without primary antibody or with an irrelevant primary antibody (see Materials and methods) exhibited no reactivity. Bar = 30  $\mu$ m.

receiving LNG-IUS. Treatment with progestagen abolished eNOS immunoreactivity in glandular epithelial cells in all of the patients treated with the LNG-IUS and in three of those treated with norethisterone (Figure 2A). Similarly, treatment with the LNG-IUS caused an attenuation or loss of iNOS immunoreactivity within glandular epithelium. By contrast, weak iNOS immunoreactivity appeared in the decidualized stromal cells after exposure to LNG-IUS (Figure 2B).

#### **NOS immunoreactivity in stromal cell of first trimester decidua**

Endothelial NOS-like immunoreactivity was localized to endothelial cells in five out of five biopsies of decidual tissue in which eNOS localization was carried out. Localization of iNOS was examined on 10 decidual biopsies using both the murine and human iNOS antibodies. With both antibodies, weak immunoreactivity was observed in decidual stromal cells (Figure 2C) which expressed vimentin but not cytokeratin, and in vascular smooth muscle.

#### **Reverse transcription-polymerase chain reaction**

Messenger RNA for both eNOS and iNOS was detected by RT-PCR in total RNA extracted from enriched gland preparations from proliferative and secretory phase endometria (Figure 3). Sequencing of the amplified products revealed 95 and 99% homology with the published sequences of eNOS and iNOS respectively. RT-PCR for iNOS in control cultures of DLD-1 cells produced one intense band of the expected size and equivalent to that observed in isolated gland fragments.

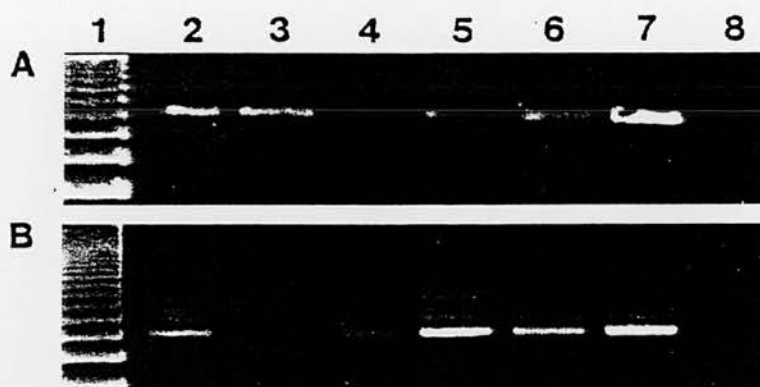
#### **Discussion**

This study has demonstrated the presence of mRNA and protein for both eNOS and iNOS in normal human endo-

metrium. Besides localization to the vascular compartment (eNOS to vascular endothelium and iNOS to vascular smooth muscle in some sections), eNOS- and iNOS-like immunoreactivity and mRNA were detected in endometrial glandular epithelium. Whilst the intensity of immunostaining varied markedly between individuals, there was no clear relationship with either the stage of the menstrual cycle at which tissue were obtained, or objectively-measured menstrual blood loss. Endothelial NOS or iNOS mRNA or protein were not found in endometrial stroma, but iNOS immunoreactivity was present in decidualized stroma in specimens obtained after the administration of synthetic progestagen and in the first trimester of pregnancy.

In our previous work which assessed immunoreactivity in six women using a different monoclonal antibody raised in mice against bovine eNOS, staining was detected in endometrial stroma and myometrial blood vessels, with weak staining in glandular epithelium in secretory endometrium (Telfer *et al.*, 1995). The present study with an anti-human eNOS monoclonal antibody revealed a consistent pattern of eNOS-like immunoreactivity in vascular endothelium and glandular epithelium. Localization of eNOS protein to glandular epithelium is supported by the distribution of NADPH diaphorase activity (Telfer *et al.*, 1995), the detection of mRNA for eNOS in glandular epithelium by Northern analysis (Tseng *et al.*, 1996) and RT-PCR as described here.

Demonstration of iNOS-like immunoreactivity in endometrial glandular epithelium was also supported by the detection of iNOS mRNA in glandular epithelial cells by RT-PCR. In addition, Tseng *et al.* (1996) detected mRNA for iNOS in enriched endometrial gland preparations by Northern blot analysis. However, in contrast to eNOS, iNOS message was only found in glandular epithelia derived from menstrual endometrium; the different results obtained with RT-PCR and



**Figure 3.** Reverse transcription–polymerase chain reaction (RT–PCR) for (A) endothelial nitric oxide synthase (eNOS) and (B) inducible nitric oxide synthase (iNOS) using total RNA extracted from freshly isolated gland fragments. Lanes 2–4 represent the proliferative phase and lanes 5–7 the secretory phase. Lane 1 is a 123 bp ladder and lane 8 is a negative control from which cDNA was omitted. Five out of six specimens produced an intense band of the appropriate size (486 bp) for the eNOS amplicon. One proliferative phase specimen (lane 4) produced a much fainter band of the same size. RT–PCR with iNOS primers produced a major band of the predicted size (375 bp) in five out of six specimens. This product had 99% sequence homology with the published human iNOS sequence. Products of higher molecular weight were also observed in three secretory phase specimens although their identity is unknown.

Northern blotting might be due to the different sensitivities of these two techniques.

Although not found in stromal cells throughout the normal cycle, iNOS-like immunoreactivity was present in stromal cells which had undergone decidualization following either the administration of synthetic progestagen *in vivo*, or the establishment of pregnancy. This *in-vivo* study does not discriminate between a direct effect of progesterone on iNOS expression or a secondary effect of local paracrine mediators that are induced by progesterone. In this study we have obtained no evidence for effects of oestrogen on eNOS and iNOS expression in human endometrium. Similarly in the sheep uterus exogenous oestrogen had no effect on NOS in the endometrium although it increased  $\text{Ca}^{2+}$ -dependent NOS in the myometrium (Figuerola and Massmann, 1995).

Identification of eNOS and iNOS in human endometrium suggests that NO may be involved in the local control of uterine function. Moreover, localization of NOS to vascular and non-vascular tissues would not preclude a predominant action on the uterine vascular bed. For example, the renal tubular epithelial cells of the macula densa release NO which dilates the neighbouring afferent artery to increase the glomerular filtration rate (Wilcox *et al.*, 1992). Nitric oxide might participate in both the initiation and control of menstrual bleeding. According to Markee (1940), the onset of menstruation is preceded by endometrial regression, vasoconstriction and then vasodilatation. Nitric oxide could contribute to these events as the most potent known vasodilator. Next, along with the vasodilatory prostanoids, prostaglandin  $\text{E}_2$  and prostacyclin (prostaglandin  $\text{I}_2$ ), NO might play a role in determining the degree of menstrual bleeding (Smith *et al.*, 1981), though a relationship between the intensity of immunostaining and objectively-measured menstrual blood loss was not found in the present study. In addition, NO may play a part in the inhibition of platelet aggregation within the endometrium. During the first few days of bleeding, haemostasis is achieved mainly by vasoconstriction and not by the deposition of platelet-fibrin plugs (Christiaens *et al.*, 1980).

Thus, the endometrium is thought to heal not by clot organization and subsequent scarring, but by vasoconstriction followed by the growth of new blood vessels.

Endometrially-derived NO could also contribute to the local control of myometrial contractility (Izumi *et al.*, 1993; Buhimschi *et al.*, 1995). Much attention has focused on the part that NO might play in maintaining myometrial quiescence during pregnancy, suggesting NO administration as a therapeutic approach for the treatment of pre-term labour (see Norman and Cameron, 1996 for review). Nitric oxide also appears to relax the non-pregnant myometrium, an action which could be exploited for the medical treatment of primary dysmenorrhoea (Pittrof *et al.*, 1996).

Nitric oxide can be released from vascular endothelium by a range of stimuli, including endothelin (ET) binding to the  $\text{ET}_\text{B}$  receptor (Takayanagi *et al.*, 1991). Endothelin-like immunoreactivity is found on vascular endothelium and glandular epithelium in human endometrium (Cameron *et al.*, 1992; Salamonsen *et al.*, 1992). Furthermore,  $\text{ET}_\text{B}$  receptors are predominantly localized to the same cell type as NOS-like immunoreactivity in the endometrium, namely glandular epithelium (Collett *et al.*, 1996). The precise relationships between NO, ETs and other locally-produced mediators of endometrial function remain to be elucidated.

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